TOWARDS AN IMMUNE CORRELATE OF PROTECTION FOR *SHIGELLA* INFECTION:

SHIGELLA-SPECIFIC PROTECTIVE IMMUNE PROFILES INDUCED AFTER PARENTERAL IMMUNIZATION OR ORAL CHALLENGE WITH TWO DIFFERENT *SHIGELLA* SEROTYPES

by

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ABSTRACT

Diarrheal diseases are a leading cause of global morbidity and mortality with an especially high disease burden in children under the age of 5 living in developing countries. *Shigella* species are a leading contributor to this disease burden and is also a significant antimicrobial resistance threat. While vaccination remains one of the most viable options to significantly reduce the disease burden caused by *Shigella* species, immune correlates of protection have yet to be defined for *Shigella*-specific immune responses. This dissertation aims to investigate *Shigella* specific protective immune mechanisms by: 1) characterizing the immune responses induced after oral challenge with *Shigella flexneri* 2a or *Shigella sonnei*, 2) identifying immune responses associated with protection from shigellosis after parenteral immunization, and 3) investigating similarities and differences in protective immune profiles associated with parenteral immunization with a *S. flexneri* 2a bioconjugate, oral exposure to *S. flexneri* 2a and, oral exposure to *S. sonnei*.

Two different controlled human infection models (CHIMs) conducted in North American adult populations were used to accomplish the research for this dissertation. The first CHIM investigated immune responses pre- and post-infection with increasing doses of virulent *S. sonnei*. The second CHIM investigated immune responses associated with protective efficacy after parenteral immunization with a *S. flexneri* 2a bioconjugate vaccine. Subjects were immunized intramuscularly twice and challenged orally with

virulent *S. flexneri* 2a. The placebo cohort from this second CHIM was used to investigate immune responses pre- and post-challenge with *S. flexneri* 2a without prior intervention.

Protective efficacy in vaccinated subjects strongly correlated with lipopolysaccharide (LPS)-specific serum IgG and IgG1 responses. The bioconjugate also induced LPS-specific gut-homing B cells associated with protective efficacy, indicating the vaccine induced immune effectors functioning at the site of intestinal infection. Subjects challenged with *S. flexneri* 2a or *S. sonnei* without prior intervention demonstrated robust mucosal responses, increases in *Shigella*-specific serum antibodies, as well as functional and memory B cell antibody responses. Pre-challenge LPS-specific memory B cell IgA responses were associated with a reduced risk of shigellosis post-challenge with both serotypes.

An important finding from the current research was the difference in immune response profiles post-infection with *S. sonnei* or *S. flexneri* 2a. Prior *Shigella* infection has demonstrated protection from subsequent infection in a serotype-specific manner, indicating that serotype-specific immune responses post-infection may serve as immune correlates or surrogates of protection. In the current research, infection with *S. sonnei* induced robust mucosal responses however, little variance was observed in the systemic responses across disease outcome. In contrast, oral challenge with *S. flexneri* 2a induced a more balanced systemic and mucosal response. Results indicate that protection from each serotype could require different protective immune mechanisms. As a quadrivalent vaccine including *S. sonnei* and *S. flexneri* 2a would be required to significantly reduce the disease burden, different protective immune mechanisms could have important impacts

on *Shigella* vaccine design, as well as the epidemiological patterns of *Shigella* species. Although the two CHIMs were not appropriately powered to fully define the protective immune profiles, several molecular and pathogenic differences in *S. sonnei* could explain some of the observed differences. The results presented herein warrant further investigations into *Shigella*-specific immune profiles either in upcoming or retrospective *Shigella* CHIMs.

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CHAPTER 1. INTRODUCTION

1.1. SPECIFIC AIMS

This dissertation uses two *Shigella* controlled human infection models to evaluate the following three specific aims:

1) To characterize the immune responses induced in humans after controlled infection with *Shigella sonnei* 53G.
2) To characterize the immune responses in a human challenge study after parenteral immunization with a *Shigella flexneri* 2a bioconjugate vaccine, as well as after controlled infection with *Shigella flexneri* 2a 2457T.
3) To investigate and describe protective immune profiles for *Shigella* infection in order to guide the rational design and assessment of *Shigella* vaccines.

1.2. OVERARCHING HYPOTHESIS

Multiple different immune profiles exist to provide protection against *Shigella* infection, depending on the route of exposure or *Shigella* strain, rather than a singular immune correlate of protection.

1.3. DISSERTATION ORGANIZATION

This dissertation is presented in six chapters. Chapter 1 focuses on providing an introduction of the research focus and specific aims. Chapter 2 uses current literature to

provide a background on diarrheal diseases and their public health significance. Chapter 2 then moves into a detailed background on *Shigella* infection and pathogenesis, as well as immunological correlates of protection and proposed mechanisms of protection from *Shigella* infection. Chapters 3, 4 and 5 correspond to Specific Aims 1, 2 and 3 and are three separate manuscripts that have been, or will be, submitted to peer reviewed journals. The first manuscript (Chapter 3) has been accepted for publication and describes the immune responses in North American adults pre- and post-oral challenge with *Shigella sonnei*. The second manuscript (Chapter 4) has been submitted for publication and describes the immune responses in North American adults pre- and post-parenteral immunization with *Shigella flexneri* 2a bioconjugate vaccine. As the primary outcome of the study used in Chapter 4 was to assess the efficacy of the bioconjugate vaccine, the study also included a population of non-vaccinated "placebo" subjects that were orally challenged with *Shigella flexneri* 2a. The third manuscript (Chapter 5) uses both studies described in Chapters 3 and 4 to compare and contrast the immune responses induced after parenteral immunization versus oral challenge. Chapter 5 also investigates differences in immune responses in subjects orally challenged with different *Shigella* species. Finally, Chapter 6 contains the conclusions of the current research as well as the public health implications and next steps for future research.

CHAPTER 2. BACKGROUND

2.1. DIARRHEAL DISEASES AND PUBLIC HEALTH

According to the World Health Organization (WHO), deaths due to diarrheal diseases are the eighth-leading cause of global mortality with approximately 1.6 million deaths per year.[1] In addition to the high mortality estimates, diarrheal disease associated-morbidity is ranked as the sixth leading cause of global disability adjusted life years.[2,3] While global morbidity and mortality estimates have decreased over the past decade, low to middle income countries (LMICS) with limited access to proper sanitation, health care, and safe water and food sources remain disproportionately affected by diarrheal diseases.[4] LMICs in sub-Saharan Africa and Southeast Asia are particularly affected, with diarrheal diseases named one of the leading causes of years of lost life.[5] Disease progression and severity is often further exacerbated in these areas due to poor nutritional and health status of infected persons.[6-10]

Children under 5 years of age living in LMICs have been identified as an especially vulnerable population, accounting for approximately 27% of diarrheal disease-associated global mortality making it the second leading cause of death in this age group .[1,11-13] The effects of diarrheal diseases in children under 5 extends beyond the acute illness and mortality with their demonstrated negative impacts on child development.[6-8,10,14] Repeated, non-fatal enteric infections can lead to reduced gut permeability causing not only an increased susceptibility to additional enteric infections but also a reduction in intestinal absorption of nutrients.[7,15] Reducing the ability to absorb nutrients in a

population where access to nutrient rich food sources may already be problematic, can lead to a constant state of malnutrition.[7,15] Such extended periods of malnutrition during childhood development have been associated with reduced height-for-age Z scores, as well as an intelligence quotient decrement of up to 10 points.[7,9,16]

Children impacted by this physical and cognitive stunting are estimated to be at a higher risk of both mortality due to other infectious diseases[17,18] as well as adulthood obesity and its associated comorbidities.[7] Additionally, enteric infections have been shown to increase the risk of developing inflammatory bowel disease (IBD),[19,20] resulting in symptoms that may require life-long treatments. Altogether, these developmental deficits can result in reduced human capital as exemplified by a reduction in economic productivity, overall intelligence (as measured by IQ) and progression of communities.[7,21] For these reasons, WHO and the United Nations International Children's Emergency Fund have prioritized the treatment and prevention of diarrheal diseases with the goal of ending preventable deaths due to diarrheal diseases by the year 2025.[22]

2.2. *SHIGELLA* EPIDEMIOLOGY AND POST-INFECTIOUS SEQUALAE

While many pathogens contribute to the diarrheal disease burden observed in children under 5 years old living in LIMCs, a select few pathogens have been identified as being responsible for 70-80% of the diarrheal disease-associated morbidity and mortality.[11,23-26] During an investigation of the pathogen-specific attributable fractions for moderate to severe diarrhea (MSD) in children under 5 years of age living in several sites across Africa and Asia, the Global Enteric Multicenter Study (GEMS) revealed that the majority of MSD cases in this population were caused by rotavirus, followed by *Cryptosporidium*, *Shigella* and heat-stable producing enterotoxigenic *Escherichia coli*.[24] While the GEMS employed commonly used enteric-specific pathogen identification methods, such as conventional culture techniques and microscopy, these methodologies have varied sensitivities across different pathogens, potentially leading to inaccurate disease burden estimates. For these reasons, a re-analysis of the GEMS samples was conducted using a highly sensitive and reproducible quantitative real-time PCR assay which revealed *Shigella* species as having the highest diarrheal disease attributable fraction.[26] Members of the genus *Shigella* are gram-negative, rod-shaped, non-motile, facultative anaerobes transmitted via the fecal-oral route that have been traditionally associated with bacillary dysentery. Recent evidence has also implicated *Shigella* species as being a significant cause of watery diarrhea,[27] making *Shigella* infection responsible for upwards of 83.2% of diarrheal cases in children between the ages of 1-5.[26,27]

While focus is often placed on the *Shigella*-specific disease burden associated with children living in LMICs, shigellosis is also considered a risk for those traveling to

endemic regions, including deployed military personnel.[28] The importance of *Shigella* infection in military populations was further emphasized when diarrheal diseases caused by bacterial pathogens, including *Shigella*, was ranked as the leading infectious disease threat to deployed military troops.[29] While *Shigella* infection can increase the risk of incapacitation or hospitalization due to the severe acute illness it causes, additional risks exist for military personnel.[28,30] Acute stress, such as that experienced during deployment, can have detrimental effects on the gut-brain axis, potentially leading to alterations of the gastrointestinal barrier and eventually causing gastrointestinal barrier dysfunction.[31-35] A pattern similar to that observed in malnourished children living in LMICs soon begins to emerge in these military populations as gastrointestinal barrier dysfunction causes a subsequent increased susceptibility to enteric infections and reduced absorption of nutrients.[33,35]

The risk of receiving an IBD diagnosis after repeated enteric infections is also of concern for military populations as IBD is further complicated by its association with an increased risk of post-traumatic stress disorder (PTSD) upon return from deployment.[36] The cycle of stress, increased disease susceptibility and increased risk of an IBD diagnosis is not only limited to deployed military personnel but extends to others who may be traveling to *Shigella* endemic LMICs in support of humanitarian efforts, or to act as peacekeepers in areas of conflict.[29,37,38] Each outlined scenario requires intense concentration as well as rapid response times and *Shigella* infection may affect either acute or chronic cognitive functions both during, and after infection. These negative cognitive outcomes in military populations draw similarities to LMIC children and the

strong link between physical/cognitive stunting and negative health outcomes in this population.[18] Together, these observations provide further evidence for the relationship between the gut-brain axis and potential cognitive impairments associated with *Shigella* infection and emphasize the importance of further investigations to fully understand this relationship.[31-34]

Additional *Shigella*-specific post-infectious sequalae have been identified as a risk across all populations, regardless of nutritional status or gastrointestinal barrier health. Reactive arthritis, an inflammatory autoimmune disorder, is identified in approximately 6% of individuals post-infection with *Shigella* species.[39,40] The genetics of the association between reactive arthritis and *Shigella* infection has been attributed to the presence of human leukocyte antigen molecule B27 (HLA-B27). HLA-B27, a gene that has also been associated with other inflammatory auto-immune disorders such as ankylosing spondylitis,[41] codes for a class I major histocompatibility complex on the surface of all nucleated cells and is responsible for presenting antigenic peptides to CD8+ T lymphocytes. The presence of HLA-B27 is associated with a higher percentage of individuals receiving a diagnosis of reactive arthritis post-*Shigella* infection to approximately 80%.[39]

Shigella-associated post-infectious sequalae are not only a concern for those living in, or traveling to, LMICs where *Shigella* species are endemic. Higher income countries, such as those across North America and Europe, have reported an increased frequency of *Shigella* outbreaks over the past decade. The United States Center for Disease Control (CDC) National Outbreak Reporting System has reported an especially

high number of outbreaks across Texas, Ohio, Pennsylvania and California.[42] Additionally, the European Centre for Disease Prevention and Control has observed an increase in shigellosis outbreaks associated with an influx of refugees emigrating from countries where *Shigella* is endemic.[43] Increased shigellosis outbreaks in healthcare and daycare settings have also been reported with some outbreaks continuing for months.[44-47] Sustained transmission in health/daycare settings is likely attributable to the fact that *Shigella* species are highly infectious, with as few as 100 bacteria reported to cause disease,[48-51] subsequently allowing for rapid spread in such settings.[44]

Shigella infections and outbreaks also occur when developed nations experience breakdowns of infrastructure, as exemplified by the 2014 water crisis in Flint Michigan. After a change in water sources, officials in Flint, MI failed to ensure proper infrastructure of piping systems supporting the new water source. Without the proper infrastructure in place, water sources were soon contaminated with serious health hazards, including heavy metals and infectious pathogens. A large outbreak of shigellosis was reported in surrounding MI counties with over 100 cases of *Shigella* infection reported.[52,53] Furthermore, natural disasters and civil war crises causing infrastructure breakdowns or population upheaval also increases the risk of shigellosis outbreaks with multiple *Shigella* epidemics attributed to such instances.[54]

Currently, treatment for *Shigella* infection involves a course of antibiotics and potentially oral rehydration solution. Although antibiotics are generally effective against shigellosis, access to antibiotics may be limited in LMICs and increasing rates of resistance to first line (ciprofloxacin), as well as alternative antibiotics, including other

fluoroquinolones, have been reported.[55-59] The CDC has classified drug-resistant *Shigella* species as a serious public health threat with high potential to worsen in the coming years and suggests increased public health monitoring and prevention activities.[60] Additionally, the WHO has placed *Shigella* on a global priority list of antibiotic-resistant bacteria in an effort to help prioritize the development of alternative measures to treat or prevent shigellosis.[55,56] For these reasons, in addition to *Shigella*-associated post-infectious sequalae, *Shigella* species have garnered the attention of multiple international agencies and stakeholders who recommend the prioritization and acceleration of vaccine development to prevent shigellosis.[55,61] The prioritization of *Shigella* vaccine development was further emphasized by the WHO during a recent workshop convened in order to investigate reduced timeline pathways to licensure and eventual funding from the Global Alliance for Vaccines and Immunization.[55,56]

2.3. *SHIGELLA* PATHOGENESIS

Shigella species are divided into four serogroups (*S. flexneri*, *S. sonnei*, *S. dysenteriae* and *S. boydii*) and, with the exception of *S. sonnei*, each serogroup is further divided into multiple serotypes (N=54, Table 2.1) based on the O-polysaccharide (OPS) structure of the lipopolysaccharide (LPS).[62] *S. boydii* is relatively uncommon while *S. dysenteriae* is traditionally associated with epidemics and pandemics, leaving the *S. flexneri* and *S. sonnei* serogroups responsible for the majority of the global disease burden.[24,26,54] Interestingly, infection with *S. sonnei* is most commonly associated with higher income countries as compared to LMICs; however, this paradigm does appear to be shifting.[18,63-65] *Shigella* infection in humans is characterized by the ability of the bacteria to invade the mucosal epithelium, replicate intracellularly and spread intercellularly, all while evading host immune responses. Successful infection is accomplished with the help of virulence proteins encoded for on a circular plasmid which is highly conserved across all virulent *Shigella* species. While the LPS biosynthesis genes are typically coded for on the chromosome, *S. sonnei* is unique in that the virulence plasmid of this serogroup also houses the LPS biosynthesis genes.

Table 2.1. Listing of 54 known species and serotypes within the genus *Shigella*.

Serogroup	Species	Serotypes	Total
A	*Shigella dysenteriae*	1 – 15	15
B	*Shigella flexneri*	1a, 1b, 1d, 2a, 2b, 3a, 3b, 4a, 4av, 4b, 5a, 5b, 6, 7a, 7b, X, Xv, Y, Yv	19
C	*Shigella boydii*	1 – 19	19
D	*Shigella sonnei*	1	1

Adapted from Muthuirulandi Sethuvel *et al*. (2017) and includes more recent serotypes proposed by the authors.[62]

In order for shigellae to reach its site of infection, the large intestine, the bacteria must first pass through the stomach and small intestine. Highly effective acid resistance systems allow the bacteria to safely travel through the acidic environment within the stomach.[66,67] Once the small intestine is reached, shigellae make use of host bile salts to induce the up-regulation of bacterial survival genes.[68] During this time, *Shigella* species also down-regulate the expression of host antimicrobial peptides, such as LL-37 and human β-defensin-1, which are released at mucosal surfaces in order to act as barrier effectors.[69] Together, these virulence factors allow the safe passage of *Shigella* species through the stomach and small intestine, and ultimately contribute to the low infectious dose observed with *Shigella* species.[66,67]

Shigella species show high selectivity to the heavily glycosylated mucins located in the human colon, allowing them to begin creating their environmental niche once the site of infection has been reached.[70] The first step of host cell invasion requires the assembly of a Type 3 Secretion System (T3SS), a process triggered at 37˚C. Assembly of the T3SS begins with the formation of a seven-section basal body which spans the bacterial inner and outer membrane, as well as the periplasm and peptidoglycan layer. The basal body is composed of several Mxi and Spa virulence proteins and is built with the help of Ipg proteins which act to stabilize the basal body and anchor it within the periplasmic leaflet of the outer membrane.[67,71]

After completion of the basal body, formation of the secretion needle can begin. The needle is largely composed of MxiH, with MxiI creating a needle base spanning the outer membrane and periplasm.[67,71] Interestingly, needle length, which is regulated by

Spa32, is thought to have coevolved with the OPS of each *Shigella* species in order to allow for optimal infectivity while still providing protection from innate immune effectors.[72] Once the needle has been created at its optimal length, the final step of forming the needle tip can begin. The T3SS needle tip is composed of translocator proteins IpaB, IpaC and IpaD, each of which is essential in the early stages of host cell invasion. IpaB and IpaC await translocation signals in the bacterial cytoplasm, bound and separated by the chaperone protein IpgC. IpaD also resides in the cytoplasm, however, as IpaD has self-chaperoning activity, it does not make use of a chaperone protein. Once translocation signals are received, IpaB and IpaC interact upon their release from IpgC, and all three Ipa proteins are translocated to the needle tip, forming a plug to prevent the excretion of effector proteins prior to receiving the appropriate signals.[67] With a completely formed T3SS, the bacteria are ready to begin host cell invasion.

While *Shigella* species carry out their replicative life cycle within intestinal epithelial cells, they are unable to invade the lumen exposed apical side of these highly polarized epithelial cells as they are lined with several defenses, including a thick glycocalyx, competing commensal bacteria and their by-products, as well as host antimicrobial substances. Therefore, *Shigella* species must gain access to the basolateral side of intestinal epithelial cells and they do so by inducing their own uptake into specialized epithelial cells. These specialized epithelial cells, known as M cells, are responsible for transporting antigens from the intestinal lumen to the underlying lymphatic tissues called Peyer's patches. Multiple innate and adaptive immune cells reside in Peyer's patches waiting to sample antigens that are transcytosed via M cells.

Shigellae take advantage of M cell function and specifically target these cells in order to gain access to the lamina propria.

Shigella species target areas of cholesterol accumulation on host cell membranes, known as lipid rafts, to begin the process of induced transcytosis. The virulence protein IpaB binds cholesterol with high affinity and causes the aggregation of additional lipid rafts which ultimately function as a docking station for the bacteria. Once docked, IpaB also binds host CD44 while the IpaB/IpaC/IpaD complex binds host integrin α5β1, subsequently inducing M cell cytoskeleton remodeling and beginning the process of engulfing the docked *Shigella*. While the binding of Ipa proteins to CD44 and α5β1 receptors begins the process of host cell cytoskeleton remodeling, bacterial uptake is dependent on the ability of the *Shigella* to translocate six effector proteins into the host cell which function to further reorganize host cell cytoskeleton and complete the process of bacterial engulfment. Effector proteins VirA, IpgB1 and IpgB2 are responsible for microtubule destabilization while IpgB2 and IpaA work together to polymerize or depolymerize host cell actin as needed in order to close the phagocytic cup.[67,71]

Once completely engulfed by the M cell, shigellae are transcytosed from the apical side of the epithelium to the basolateral side where they are deposited into the Peyer's patches. Macrophages are typically the first cells to come into contact with shigellae on the basolateral side and immediately begin to phagocytize the bacteria and form a phagosome. Shigellae enhance this process of phagocytosis using the same mechanisms employed to induce its uptake via M cells. Typically, macrophages then fuse the phagosome with a lysosome in order to kill the engulfed bacteria. However, shigellae

make use of phagosome membrane lipid rafts and bacteria-derived effector proteins to prevent the formation of a phagolysosome. IpaC plays an essential role in disrupting the integrity of the phagosome phospholipid membrane, allowing shigellae to quickly degrade the membrane and efficiently escape from macrophage phagosome within 15 minutes of uptake.[67,71]

Upon escape from the phagosome, the strong innate inflammatory response observed during *Shigella* infection begins to unfold as the bacteria start the process of inducing pyroptotic macrophage cell death. Multiple effector proteins, including IpgD and MxiE, provide anti-apoptotic signaling in order to override host cell-induced apoptosis, therefore ensuring the progression through the pyroptotic inflammatory cell death pathway. Simultaneously, OspF, OspG and IpaH interfere with MAPK and NF-κB signaling in order to prevent the infected macrophage from making other host innate immune cells aware of the infection and subsequently working to kill the infected macrophage. At this stage, IpaB initiates the process of pyroptosis by directly binding to and activating Caspase-1. Activated Caspase-1 proteolytically cleaves pro IL-1β and pro IL-18 cytokines to their active proinflammatory forms which are released from the macrophage upon pyroptotic cell death. Additionally, the shigellae contained within the macrophage are released upon cell death and are now free to invade the basolateral surface of the intestinal epithelial cells.

Release of IL-1β and IL-18 into the lamina propria upon macrophage cell death induces a strong inflammatory response as these cytokines act as chemoattractants, recruiting natural killer cells and neutrophils to the site of infection.[67,71] Natural killer

cells work to further activate host immune responses through the secretion of IFN-γ and, while neutrophils eventually contribute to controlling *Shigella* infection, they exacerbate the inflammatory response during the initial stages of infection. The influx of neutrophils can damage the integrity of the epithelial cell lining as they insert themselves between epithelial cell tight junctions in an effort to find and kill the shigellae. While neutrophils are inserting themselves between the epithelial cells, shigellae are also able to pass through the widened spaces in the epithelial cell lining and gain access to the lamina propria and basolateral side of epithelial cells, without having to use M cells.[67,71]

Epithelial cell invasion and subsequent escape from epithelial cell phagosomes occur in the same manner as described for M cell invasion and macrophage phagosome escape. Upon release from epithelial cell phagosomes, the bacteria do not induce cell death, but rather begin their process of replication and intercellular spread. Shigellae quickly replicate within host cells and reach a critical mass where additional space is needed. While shigellae do not possess structures such as flagellin, which allow bacterial motility, they are able to make use of host cell actin in order to move about host cells. Virulence protein VirG plays a vital role in the movement of shigellae by localizing to one pole of the bacteria and recruiting a complex of N-WASP and Arp2/3. The N-WASP-Arp2/3 complex serves as an actin nucleator, polymerizing host cell actin and creating actin tails which propel the bacteria forward. VirA works at the opposite pole of the bacteria to degrade host cell α-tubulin, essentially clearing a pathway forward for the bacteria. During movement of the bacteria around the host cell, shigellae will eventually interact with the epithelial cell wall and begin the process of intercellular spread as they

continue to propel their forward motion until they are able to push through the wall of the current cell into an adjacent epithelial cell. At this point, the shigellae are contained within a new epithelial cell phagosome allowing them to begin the process anew.[67,71]

During the process of bacterial replication and intercellular spread, epithelial cells also contribute to the intestinal inflammatory response of *Shigella* infection. Intracellular spread of the bacteria and exposure of epithelial cells to high amounts of cytosolic bacterial LPS induces inflammasome activation through the non-canonical pathway after TLR-4 recognition of LPS and subsequent activation of caspase-4. Additionally, host cell NOD-1 receptors recognize bacterial peptidoglycans and activate NF-κB signaling pathways, causing the release of chemoattractant IL-8 into the lamina propria. IL-8 works to recruit additional neutrophils to the site of infection in order to help manage the infection. Eventually, the immune system is able to control the intercellular spread of the bacteria but not before a tremendous amount of inflammation and damage to the epithelial cell lining occurs, all of which results in the bloody mucoidal stools and colonic ulceration often observed in *Shigella* infection.[67,71]

While most *Shigella* species use the same virulence factors to cause infection in a similar manner, a few serotypes set themselves apart from the rest of *Shigella* serotypes. *S. dysenteriae* 1 has traditionally been known as the only *Shigella* serotype to harbor the Shiga toxin; one of the most potent toxins across all bacterial species.[73,74] More recently however, the Shiga toxin has also been discovered in *Shigella flexneri* serotypes, indicating that *S. dysenteriae* 1 may no longer be the only serotype to make use of this toxin. The Shiga toxin is an αβ toxin which works to inhibit host cell protein synthesis

causing a breakdown of the epithelial cell lining and subsequent hemorrhaging.[73,74] This toxin is associated with severe life-threatening disease and is one of the reasons *S. dysenteriae* is often associated with epidemics and pandemics.

Shigella sonnei is another unique serotype for several reasons. *S. sonnei* encodes for a Type 6 Secretion System (T6SS) which is used to directly compete with and kill, not only other *Shigella* species, but also other enteric bacteria including *E. coli* species.[75] Interestingly, the T6SS of *S. sonnei* has been cited as one of the reasons that *S. sonnei* has been able to rise to dominance in higher income countries: individuals in LMIC settings have gained natural immunity to *S. sonnei* through frequent exposure to *Plesiomonas shigelloides*, a bacterium which possesses a homologous LPS structure to *S. sonnei* and is found often in low-resource settings with poor infrastructure.[76] Those living in more developed nations do not have the opportunity to gain this cross-reactive natural immunity as infrastructure and sanitation measures in these countries have resulted in a low prevalence of *P*. *shigelloides*. Without this natural immunity in higher income countries, *S. sonnei* has been able to out-compete other *Shigella* serotypes with the help of its T6SS. Another unique attribute of *S. sonnei* is its adeptness in acquiring antimicrobial resistance genes as compared to other *Shigella* serotypes, allowing them the opportunity to persist during infection, even during treatment.[64] Finally, *S. sonnei* has found a protective niche in the ubiquitous *Acanthamoeba castellanii* amoebae which engulf *Shigella* species in the environment and as a result, the provide *Shigella* species with protection from sanitation processes, such as chlorination. While many *Shigella*

serotypes, such as *S. flexneri*, are lethal to *A*. *castellanii, S. sonnei* is not, allowing it to take full advantage of this protective niche and hide from unfavorable conditions.[77]

2.4. IMMUNOLOGICAL CORRELATES OF PROTECTION

2.4.1. Immune Correlates and Their Use in Vaccine Development

A correlate of protection (CoP) is defined as an immunological parameter that is statistically correlated with, and therefore predictive of, vaccine efficacy.[78] CoPs are a vital aspect to understanding pathogen-specific protective immunity a parameter that can ultimately guide the rational design and assessments of candidate vaccines. Furthermore, a defined CoP can be used to accelerate vaccine licensure, especially in the case of multivalent vaccines where changes or additions to pathogen serotypes are required.[79] CoPs are also useful post-licensure as well to determine an individual's pathogen-specific immune status, or to assess a newly manufactured lot of an established vaccine to ensure comparability of the new lot with previously manufactured lots.[79]

Before a CoP can be investigated however, the primary clinical endpoint must be clearly defined.[80] The primary endpoint is the outcome targeted for prevention and can vary across different pathogens, trial settings and target populations. For example, a primary outcome of providing sterilizing immunity (i.e. preventing infection) in an infant population may require a different CoP as compared to a target outcome of only preventing clinical signs and symptoms (i.e. preventing disease) in an adult population. If the desired outcome is to prevent disease, the clinical signs and symptoms must be further defined to understand the level or severity of disease being targeted for prevention. Preventing disease could be stringently defined as the prevention of any visible clinical signs or, the perhaps the goal is prevention of hospitalization or,

prevention of death; each of which may have a different CoP and could vary by vaccine and pathogen type.[80] The nuances of defining the primary outcome are exemplified by CoPs associated with the measles vaccine: serum IgG titers ≥200 milli-international units per ml (mIU/ml) provide sterilizing immunity and therefore prevent infection, whereas titers between 120-200 mIU/ml provide protection against clinical signs of disease but not infection and finally, titers <120 mIU/ml do not provide protection against infection or disease.[80]

A CoP can be further divided into two categories: a mechanistic correlate of protection (mCoP) or a non-mechanistic correlate of protection (nCoP).[78] The main difference between mechanistic and non-mechanistic CoPs is that mCoPs are a mechanistic and therefore causal protective immune parameter often functioning at the site of infection to provide protection, while nCoPs, although they are a predictor of protection, are not the causal means of the protective immune response. A CoP is either an mCoP or a nCoP but cannot be both;[78] however, nCoPs can act as surrogate measures for mCoPs. Although it is preferable to define and implement the use of a mCoP rather than an nCoP, it is not always feasible or practical if the mCoP is difficult to obtain or measure, as in the case of the Zoster vaccine.[78] Both serum antibody responses and cellular responses post-vaccination with Zoster are statistically correlated with protection and therefore both are defined as CoPs; however, when considering the biology of the pathogen inside the host, cellular responses are likely working at the site of infection to prevent disease. Furthermore, the statistical association of cellular responses and protection is more robust as compared to the association of serum antibody responses

and protection. Together, these data can be used to define cellular responses as the mCoP and serum antibody responses as the nCoP. In the case of Zoster, it is simpler and more efficient to measure antibody responses in serum as compared to cellular immune responses therefore, the nCoP is typically used in vaccine efficacy assessments.[78]

While defining a CoP is an important goal in vaccine development, one must consider the possibility of multiple CoPs acting together as co-correlates to synergistically provide protection.[78,80] This was exemplified in vaccine trial assessing the efficacy of a live-attenuated intranasal influenza vaccine. Subjects in this trial were administered the live-attenuated flu vaccine or placebo, and subsequently challenged with live influenza virus. The clinical endpoint for this study was defined as a reduction in viral shedding and hemagglutination-inhibiting (HAI) serum IgG and nasal wash IgA immune responses were determined. Subjects that did not have either HAI serum IgG or nasal IgA shed the virus 63% of the time whereas subjects that had both immune parameters shed the virus 3% of the time. Interestingly, subjects with only HAI serum IgG shed the virus 15% of the time while subjects with only HAI nasal IgA shed the virus 19% of the time.[80,81] Although viral shedding was significantly reduced with either HAI serum IgG or nasal IgA, an additional reduction in viral shedding to 3% clearly demonstrated the synergistic effects of having both serum IgG and nasal IgA.[80,81] Therefore, when investigating CoPs associated with a clinical endpoint, it is essential to have a full understanding of organism pathogenesis, as well as to investigate as many immune responses/parameters as possible.

Once the immune parameters are defined, levels of these parameters that are associated with protection should be investigated to determine if they can be used as absolute or relative CoPs. An absolute CoP is defined as a specific quantity or level of the CoP that provides protection in a binary manner close to 100% of the time (i.e. protected at a certain level or not protected below that level).[80] The hepatitis B vaccine has a well-defined absolute CoP of serum antibody levels ≥10 mIU/ml considered protective while anything below this level is considered not protective.[82] On the other hand, a relative CoP usually provides protection at a certain level, however breakthroughs can occur below this threshold.[80] A relative CoP is exemplified in an influenza vaccine trial where approximately 70% of subjects with a HAI serum IgG titer of ≥40 were protected, however, as the titer increased or decreased, the percent of protected subjects correlated with HAI titer.[83] Although absolute CoPs are ideal, relative CoPs are more commonly observed as CoPs are often best described as a curve rather than as an absolute threshold.[80]

Investigation of CoPs must take into account multiple factors such as the type of vaccine, disease pathogenesis, population genetics or epigenetics as well as the setting used for investigational studies versus the target population for the vaccine. Each of these factors can influence the interpretation of CoP investigations, especially in the context of determining absolute or relative levels required of a CoP.[78] Vaccine types and route of administration can greatly affect the resulting immune response and associated CoP(s). For example, the live-attenuated oral polio vaccine (OPV) induces strong secretory IgA responses as well as cellular immunity dominated by CD8+ T cells. This is in

contrast to the intramuscularly delivered inactivated polio vaccine (IPV), which induces robust serum antibody responses and CD4+ T cell immunity.[84,85] These two different vaccines and delivery routes provide different levels of protection and also have different CoPs: OPV protects against infection through secretory antibodies secreted at the site of infection while IPV prevents clinical disease through the generation of high levels of serum antibodies.[84,85] Genetics and epigenetics, as well as nutritional status, play important roles in vaccine efficacy and CoPs. A CoP determined in a healthy American adult population may not translate to children in LMIC settings with varied immune or nutritional status.[79,86] The same concept applies to immunosuppressed or immunocompromised individuals. All of these influencing and potentially confounding factors affecting CoPs must be taken into consideration when defining CoPs, especially in the context of vaccine licensure or vaccine lot comparability.[79]

2.4.2. Methods of Investigating Immune Correlates of Protection

Proposed CoPs must be thoroughly tested, ideally in the target host, in order to verify their ability to predict protection as well as to determine thresholds that may be associated with protection. While animal and *in vitro* models can be used, these types of data are not always easily extrapolated to human populations and may be misleading. Such was the case while using a mouse model to investigate rotavirus CoPs. The proposed mechanism of protection in this rotavirus mouse model was the discovery of active transport of serum IgA into bile.[87] However, as this transport does not occur

efficiently in humans, this immune mechanism would likely not provide protection in the target population, requiring further investigations using an alternative model.[87,88]

If the suspected mechanism of protection is antibody-based, the use of passive antibody administration studies or maternal-newborn observational studies can be useful tools to investigate protection provided by antigen-specific antibodies.[89,90] However, a caveat must be placed on passive administration and maternal antibody studies as the protective level of antibodies required for passive protection may not be representative of the levels required for protection after vaccination as passively administered antibodies receive additional support from the host immune system.[88]

Studying the immune responses after natural infection can be a useful guide while investigating CoPs.[91,92] Determining the specificity and magnitude of the immune response post-infection can help identify key immune parameters or antigenic targets that may significantly contribute to protection. However, unless the date of infection is known, this method of investigating CoPs is best applied in the context of pathogens that knowingly provide long-lasting immunity, such as measles. Otherwise, the protective immune parameters may no longer be measurable if infection occurred long ago.[88] Knowing the date of infection is also helpful in understanding which immune parameters are associated with acute infection versus convalescence. Likely, immune parameters determined in a convalescent state would be better candidates for CoPs. Observational studies, such as cohort or case-control studies, can be used to help ensure the date of infection is documented.[82,93] In observational studies, subjects would be enrolled, their immune status investigated and then followed up for a period of time to monitor for the

occurrence of the clinical endpoint, thus providing pre- and post-exposure immune responses as well as the date of infection.[88] Observational studies used for investigating CoPs must be well-designed to control for confounding factors, such as previous pathogen exposures, history of immune and nutritional status or genetic/regional differences in populations.

If an established controlled human infection model (CHIM) exists for the pathogen of interest, one of the best ways to investigate CoPs is with the use of human challenge/re-challenge randomized controlled trials (RCT).[88,94-97] A RCT allows for better control of potentially confounding factors and can help ensure as naïve a population as possible in order to accurately investigate protective immune parameters. Assessing the immune responses pre-challenge as well as post-primary and post-secondary challenge can provide key insights for establishing CoPs. In the case of pathogens with multiple strains, challenge/re-challenge studies can also be used to assess the potential of heterologous protection, leading to the understanding of broadly protective antigenic targets as well as determining the degree of multivalency required of a vaccine.[96] The use of challenge/re-challenge studies in the *Shigella* field have been pivotal in the understanding that protection from *Shigella* infection is serotype specific and therefore, a multivalent vaccine will likely be required.[98,99]

CHIMs are also commonly used to assess the efficacy of a vaccine candidate that has undergone appropriate safety and dose-determination studies.[88] In this setting, subjects would be enrolled, vaccinated with either the candidate vaccine or a placebo control, and subsequently challenged with the pathogen of interest. Vaccine efficacy

RCTs are an excellent resource for the investigation of protective immune parameters induced by vaccination and their association with protection from the study defined clinical endpoint.[100-102] One key point for consideration regarding vaccine efficacy CHIMs is the definition of the clinical endpoint and its use across different institutions investigating the same pathogen with different vaccine candidates. Consistent use of clinically meaningful and validated endpoints and disease outcomes is essential in accurately assessing CoPs and allows for better comparisons of different vaccine candidates. The use of a consensus clinical endpoint can assist in the comparability of studies performed at different sites which could lead to the acceleration of vaccine licensure or WHO pre-qualification.[103-105]

2.4.3. Proposed Immune Correlates of Shigella Infection

Although several of the aforementioned methodologies used to investigate CoPs have been employed in the context of *Shigella* infection, a consensus CoP has yet to be defined.[92,99,106-108] This could be due, in part, to the complicated nature of the bacteria's dynamic life cycle in the large intestine as it involves both an extracellular and intracellular phase.[67] When considering the pathogenesis of *Shigella* species, one could propose that there are potentially several protective immune mechanisms, depending on the clinical endpoint of interest. If the desire is to provide sterilizing immunity and prevent transcytosis of the bacteria from the apical side of the mucosal epithelium to the basolateral side, it is likely that antibodies secreted into the lumen would be required, either by active secretion (IgA) or passive transudation (IgG). On the other hand,

protection from mild to moderate disease could be achieved within the intestinal lamina propria via antibody mediated mechanisms, including neutralization of the bacteria on the basolateral side of the epithelium to prevent epithelial cell invasion, direct killing of the bacteria by opsonization or complement activation and, antibody dependent cellular cytotoxicity mediated by natural killer cells or monocytes. Cell-mediated immunity may play a larger role in protection from more severe disease as it could work to prevent the intercellular spread of shigellae, thus preventing or reducing the bloody and mucoidal stools resulting from colonic ulceration induced by epithelial cell death.

Studies of *Shigella* pathogenesis and CoPs have provided important insights into potentially protective antigenic targets and protective immune mechanisms. Since immunity to *Shigella* infection is serotype specific, and *Shigella* serotypes are determined by the antigenic structure of the OPS, a vaccine will likely require either the OPS or full LPS molecule in order to be protective.[92,98,109] Serotype-specific *Shigella* immunity also reveals that a multivalent vaccine will be needed to protect against the most relevant circulating *Shigella* serotypes responsible for the majority of the global disease burden.[24,26,110] In addition to the requirement of multivalency, the challenge of low to undetectable infant immune responses to polysaccharide vaccines must be taken into consideration when developing OPS or LPS based *Shigella* vaccines.[79,80,84]

Additional antigenic targets have been identified, including the highly conserved virulence proteins IpaB, IpaC and IpaD, with research suggesting that vaccines containing these targets may provide pan-protection from all *Shigella* species.[111,112] Although this concept of broadly protective antigens is interesting, heterologous challenge/re-

challenge studies have demonstrated that protection from shigellosis is only achieved after homologous re-challenge, regardless of the fact that all pathogenic *Shigella* species contain the virulence plasmid encoding for the Ipa proteins.[99] While immune responses directed against the Ipa proteins may not be protective in their own right, the inclusion of these highly conserved proteins in an OPS-containing vaccine may broaden protection to other serotypes within a particular serogroup.[113]

As *Shigella* is a mucosal pathogen, immunological dogma predicts that a mCoP for *Shigella* infection would be a certain level of mucosal antibodies.[114,115] A mucosal CoP was suggested after the completion of a vaccine trial assessing the efficacy of a live-attenuated *S. flexneri* 2a vaccine. This vaccine efficacy trial demonstrated that vaccinated subjects with ≥75 LPS-specific IgA antibody secreting cells (ASC) per 10^6 total IgA PBMCs (N=4) were protected from the clinical endpoint of shigellosis.[106] While the suggested CoP of ≥75 LPS-specific IgA ASCs fits dogmatic understanding, the study was unfortunately not appropriately powered to confirm this CoP with only 7 vaccinated subjects undergoing challenge.[79] Furthermore, the assumption was made that all IgA ASCs would be contributing to the local mucosal antibody response, however, without knowing the homing pattern of these ASCs, it is difficult to confirm if the ASCs are homing to the intestine to secrete mucosal IgA, to the bone marrow to secrete serum IgA or potentially elsewhere in the body. Nonetheless, the importance of a mucosal IgA response that could potentially serve as a mCoP has remained unquestioned within the *Shigella* field.[114,115]

Alternative methodologies, aside from measuring LPS-specific IgA ASCs, can be employed to assess the *Shigella*-specific mucosal immune response. One such method includes measurement of the specialized secretory IgA response in mucosal secretions, such as stool, urine or oral fluids.[106,116,117] As secretory IgA contains the secretory component allowing its active transport into mucosal compartments, measuring the antigen-specific secretory IgA responses can be a good representation of the mucosal immune response. Although fecal antibody responses are commonly used to measure the *Shigella*-specific mucosal immune response,[118,119] the use of fecal samples in immunoassays is fraught with issues, including problems with sample quality, poor signal to noise ratios in immunoassays, and potentially low subject compliance.[117] More recently, investigations of *Shigella*-specific mucosal responses have focused on the measurement the antigen-specific responses from B lymphocytes expressing the intestinal homing marker α4β7 integrin.[120,121] Isolating B cells that are positive for the α4β7 integrin and using these cells in immunoassays to determine the *Shigella*-specific response can act as a better measure of the mucosal immune responses induced post-vaccination or infection.

Shigella LPS-specific serum IgG has also been identified as a potential CoP; however, it remains unclear if serum IgG is acting as a mCoP or a nCoP.[122-124] The suggested mechanism of protection provided by serum IgG surrounds the concept of inducing high enough levels of *Shigella*-specific serum IgG such that these antibodies would transudate from systemic circulation into the intestinal lumen, thereby providing protection from mucosal epithelium invasion by *Shigella* species. Several clinical studies

investigating candidate polysaccharide-based conjugate vaccines have shown a correlation of LPS-specific serum IgG with protection from the clinical endpoint.[125-127] While LPS-specific serum IgG may be a CoP given the observed association with protection in these clinical studies, further investigations should be undertaken to fully elucidate if it is acting as a mCoP or a nCoP. Additionally, investigations into absolute or relative threshold levels required for protection from the clinical endpoint should be performed.

Although T cell immunity has been extensively investigated in the context of *Shigella* pathogenesis and immune evasion, there has been little evidence so far to suggest their utility as a CoP for protection from *Shigella* infection[115]. In addition to T cell immune responses and IgA ASCs, memory B cell responses are another cellular immune parameter that could serve as a CoP for *Shigella* infection. Memory B cell responses have been associated with reduced disease severity as well as protective efficacy.[115,128,129] Although *Shigella*-specific memory B cells could provide the field with a mCoP, it would be useful in this circumstance to define a nCoP to substitute for measuring memory B cell responses since the opportunity to collect enough blood volume from subjects in order to isolate cells and perform these assays may not always be available, especially as the target population for a *Shigella* vaccine is infants.

Statistical methods utilizing mathematical modeling or machine learning have also been employed to provide insight into *Shigella*-specific CoPs.[114,128] These methodologies have suggested that a combination of a mucosal and systemic response working synergistically as co-correlates could provide protection from shigellosis.

Mathematical models using previously conducted vaccine efficacy trials suggested that a LPS-specific serum IgG ELISA endpoint titer of ≥300, in addition to any degree of a LPS-specific IgA ASC response predicted nearly 100% protection in studies investigated.[114] However, as these models were derived using a low number of subjects (N = 78) after immunization with a live-attenuated *Shigella* vaccine, additional investigations should be conducted using additional subjects and different vaccine candidates.

2.4.4. Immune Profiles of Protection

As outlined earlier, defining immune correlates of protection and developing efficacious vaccines can be complicated by variances in host and environmental factors such as population genetics, age, nutritional and immune status, as well as pre-existing immunity and/or concurrent infections.[130-135] Host immune responses and the success of a vaccine is further complicated in the case of enteric pathogens since the intestinal microbiome and integrity of the gastrointestinal barrier must also be considered.[133,134,136-138] Several enteric vaccines, including a rotavirus vaccine and a cholera vaccine, have demonstrated excellent success in one population followed by dramatically reduced efficacy in another population.[139-141]

In addition to differences across host and environmental factors, defining protective immune mechanisms is further complicated by the pathogen itself. Many bacteria, viruses and parasites have the ability to quickly adapt in order to increase pathogen virulence or develop resistance to current treatments and/or prophylactics.[137,142] Several other factors, including pathogen strain or serotype, route of

infection and dose can also influence pathogen virulence and host immune response.[143-145] The combination of host/population variances, environmental influences and differences in pathogen virulence gives rise to a dynamic interplay, not only between host and pathogen, but also within the host and the immune response mounted.

Defining immune correlates of protection has long been deemed the holy grail of vaccine development and while there is no question regarding their value and importance, searching for a single immune correlate (or two co-correlates) may not take into consideration the complexities of the host immune response and host-pathogen interaction. Over the past decade, advancements in research technologies and a movement towards personalized medicine has led the fields of vaccinology and immunology towards considering a broader definition of a protective immune response.[146-148] Rapid expansions in the areas of systems biology and omics research have provided essential insights into host responses to vaccination or pathogenic challenge,[130,149,150] demonstrating that protective immunity likely requires an integrated and networked immune response profile.[151]

Immune response profiles post-vaccination or post-challenge have been described or proposed for several pathogens including human immunodeficiency virus, *Plasmodium falciparum* and yellow fever.[150,152-155] Immune response profiles have also been described for the enteric pathogen *Salmonella* Typhi after parenteral immunization with two different vaccine constructs (Vi capsular polysaccharide (Vi-PS) or Vi capsular polysaccharide conjugated to tetanus toxoid (Vi-TT)) followed by oral challenge with live *S.* Typhi.[156] Interestingly, while both vaccine constructs provided similar levels of efficacy

post-challenge, they appear to have done so in different ways. Subjects immunized with Vi-PS and protected from challenge were found to have a serum IgA-dominated response, however, protected subjects immunized with Vi-TT demonstrated an increased serum IgA response in conjunction with an increased avidity of serum IgG1 antibodies.[156]

The concept of achieving a protective immune response to the same pathogen through different immune mechanisms was also exemplified earlier by the OPV and IPV polio vaccines. While both polio vaccines are protective in their own right, each provide protection at different levels and in different ways.[84,85] Being able to achieve protective immune responses via different immune mechanisms is an important consideration in vaccine development and further emphasizes the need for thorough immune response characterization post-vaccination or post-challenge in order to investigate immune response profiles.

As *Shigella* species are enteric pathogens with over 40 different serotypes and a complex lifecycle, characterization of *Shigella*-specific immune response profiles is likely especially important. Additionally, multiple *Shigella* vaccine constructs using different routes of mucosal or parenteral delivery are currently in development[157,158] and may provide protection from shigellosis in different ways. For example, an orally delivered whole cell vaccine may induce a predominantly mucosal profile with secretory IgA working to prevent or reduce bacterial invasion of the epithelium. In contrast, the profile after parenteral immunization with an LPS-conjugate may provide protection via transudation of systemic antibodies into the lumen or increased antibody effector functions within the lamina propria. Even within similar vaccine constructs, immune

responses could vary substantially depending variances such as the use of killed or live-attenuated oral vaccines, delivery of conjugate vaccines intramuscularly versus intradermally, or even the use of adjuvants to alter the phenotype of the immune response. With no defined correlate of protection for shigellosis and multiple host immune mechanisms that could work to reduce the severity or duration of infection, protection from shigellosis is likely better described as an immune profile rather than a single immune correlate.

CHAPTER 3. THE IMMUNE RESPONSE POST-INFECTION WITH *SHIGELLA SONNEI* 53G IN A CONTROLLED HUMAN INFECTION MODEL

The data presented in Chapter 3 has been accepted for publication in the American Society for Microbiology journal mSphere:

Clarkson KA, Frenck RW, Dickey M, Suvarnapunya AE, Chandrasekaran L, Weerts HP, Heaney CD, McNeal M, Detizio K, Parker S, Hoeper A, Bourgeois AL, Porter CK, Venkatesan MM, Kaminski RW. Immune Response Characterization after Controlled Infection with Lyophilized *Shigella sonnei* 53G. *mSphere* 2020; Manuscript Accepted for Publication.

3.1. ABSTRACT

Shigella is a major cause of moderate to severe diarrhea largely affecting children <5 years old living in low- and middle-income countries. Several vaccine candidates are currently in development and controlled human infection models (CHIMs) can be useful tools to provide an early assessment of vaccine efficacy and potentially support licensure. A lyophilized strain of *S. sonnei* 53G was manufactured and evaluated to establish a dose that safely and reproducibly induced ≥60% attack rate. Samples were collected pre- and post-challenge to assess intestinal inflammatory responses, antigen-specific serum and mucosal antibody responses, functional antibody responses and memory B cell responses. Infection with *S. sonnei* 53G induced a robust intestinal inflammatory response as well as antigen-specific antibodies in serum and mucosal secretions, and antigen-specific IgA- and IgG-secreting B cells positive for the α4β7 mucosal-homing marker. There was no association between clinical disease outcomes and systemic or functional antibody responses post-challenge; however, higher LPS-specific serum IgA and IgA-secreting memory B cell responses were associated with a reduced risk of disease post-challenge. This study provides unique insights into the immune responses pre- and post-infection with *S. sonnei* 53G in a CHIM, which could help guide the rational design of future vaccines to induce protective immune responses more analogous to those triggered by infection.

3.2. IMPORTANCE

Correlate(s) of immunity have yet to be defined for shigellosis. As previous disease protects against subsequent infection in a serotype-specific manner, investigating immune response profiles pre-and post-infection provides an opportunity to identify immune markers potentially associated with the development of protective immunity and/or with a reduced risk of developing shigellosis post-challenge. This study is the first to report such an extensive characterization of the immune response post-challenge with *S. sonnei* 53G. Results demonstrate an association of progression to shigellosis with robust intestinal inflammatory and mucosal gut-homing responses. An important finding in this study was the association of elevated *Shigella* LPS-specific serum IgA and memory B cell IgA responses at baseline with reduced risk of disease. The increased baseline IgA responses may contribute to the lack of dose response observed in the study and suggests that IgA responses should be further investigated as potential correlates of immunity.

3.3. INTRODUCTION

Shigella is a significant cause of bacillary dysentery resulting in moderate to severe diarrhea (MSD) in travelers, as well as children in low- and middle-income countries (LMIC).[28,159-161] Using more sensitive molecular based methods, *Shigella* has also been shown to be a significant cause of watery diarrhea.[27] In 2016, *Shigella* was identified as the second leading cause of diarrhea-associated mortality across all age groups with an increased disease burden among children under the age of 5 years.[1,23] Children are also at risk of impaired development after repeated enteric infections.[6-8,10,14] Infection with *Shigella* species can lead to reduced gut permeability, subsequently causing a reduction in intestinal absorption of nutrients, leading to childhood cognitive and physical stunting.[7,15] Children stunted from the heavy burden of *Shigella* infection are also at higher risk of dying from other infectious diseases.[17] *Shigella* remains a high priority vaccine target for the WHO because of its high global burden and increasing antibiotic resistance.[55]

Although several vaccine candidates are in clinical development, there is currently no licensed *Shigella* vaccine. A useful tool for early assessment of vaccine efficacy and, at times utilized to support licensure, is the controlled human infection model (CHIM).[104,105,162,163] In response to recent calls for model standardization,[103,164-169] a lyophilized strain of *S. sonnei* 53G was produced using current good manufacturing processes (cGMP) and evaluated in a dose-escalation protocol to determine a dose that safely and reproducibly yielded a ≥60% attack rate for shigellosis (Appendix A). Disease

outcomes and attack rates with the lyophilized strain of *S. sonnei* 53G are described elsewhere (Appendix A).

This study offered a unique opportunity to characterize systemic and mucosal immune responses given the repeated collection of multiple samples throughout the study. Since prior disease caused by *Shigella* infection can result in protection from subsequent infection in a serotype-specific manner,[92,98,99,109,170] investigation of the immune responses associated with multiple disease outcomes post-infection could provide further insights into potential mechanistic or non-mechanistic immune correlates of protection. Investigating correlates, or surrogates, of protection can also help guide vaccine development and interpretation of results in future *Shigella* CHIMs aimed at assessing vaccine efficacy.

As previously reported, LPS-specific serum and fecal IgG and IgA responses post-challenge were not associated with challenge dose or clinical disease outcomes, including dysentery, diarrhea severity and disease severity score (Appendix A). These findings highlight potential gaps in understanding the contribution of other immunological or non-immunological protective mechanisms and may also confirm the importance of immune responses to non-polysaccharide antigens.[120,171,172]

Finally, a subset of orally challenged subjects did not exhibit disease symptoms post-challenge, regardless of dose received (567 – 1760 colony forming units (cfu)) (Appendix A). Therefore, a more comprehensive evaluation of the *Shigella*-specific immune responses at baseline and at several time points post-challenge was undertaken. Investigating potential immunological mechanisms associated with observed resistance

to infection and subsequent lack of association between immune responses and disease outcomes may also provide insights into potential correlates of protection.

3.4. MATERIALS AND METHODS

Study Design and Inoculation. The study was an open-label dose-finding CHIM designed to determine a target dose of lyophilized cGMP *S. sonnei* 53G for use in future *Shigella* CHIMs that would induce shigellosis in ≥60% of subjects as described in Appendix A. Subject inclusion and exclusion criteria are detailed in Appendix A, along with subject screening and disposition. Five days post-challenge (or sooner if clinically warranted), all subjects initiated antibiotic treatment and were discharged from the inpatient facility after producing 2 culture-negative stool samples. Subjects returned 14, 28 and 56 days post-challenge for additional blood and stool sample collections. Demographic data and disease outcome data of enrolled subjects are described in detail in Appendix A.

Blood Processing. Whole blood was collected on study days -1, 5, 7, 14, 28 and 56. Serum samples were stored at -80 ± 10˚C. Peripheral blood mononuclear cells (PBMCs) were isolated on a Ficoll gradient with Leucosep tubes (Greiner Bio-One), frozen using Cool Cells (BioCision) and stored in liquid nitrogen.

Stool Processing. Stool samples for immunoassays were collected on study days -1, 0, 3, 7 and 14, and immediately frozen at -80 ± 10˚C. Archived stool samples were thawed and processed for immunoassays by suspending 2 grams of stool in a protease inhibitor buffer (Roche cOmpleteTM EDTA-free protease inhibitor tablets, 1X PBS, 0.05% Tween-20, 0.1% BSA), vortexed and centrifuged for 30 minutes at 4˚C at ~1900 x g. Fecal extract supernatants were collected and frozen at -80 ± 10˚C. Stool samples for inflammatory marker analyses were processed in myeloperoxidase or calprotectin-

specific collection/processing kits according to manufacturer's instructions (Epitope Diagnostics) and frozen at -80 ± 10˚C.

Fecal Inflammatory Markers. Stool samples processed for inflammatory marker analyses were assayed by Enzyme-Linked Immunosorbent Assay (ELISA) to determine calprotectin and myeloperoxidase concentrations per manufacturer's instructions (Epitope Diagnostics). Inflammatory marker concentrations were interpolated from a standard curve with values below the assay limit of detection (LOD) being assigned a value of half the lowest concentration in the standard curve (½ LOD). As this was a post-hoc analysis, only stool from subjects consenting the use of their samples in future or additional investigations were used for this analysis. The investigation of fecal inflammatory marker concentrations in stool samples was the only post-hoc analyses conducted and therefore the only immune response analysis conducted on a smaller subset of subjects (N=45/56 subjects: 18 with shigellosis post-challenge and 26 without shigellosis post-challenge).

Antibodies in Lymphocyte Supernatant (ALS) Generation. PBMCs used to generate ALS were suspended in complete RPMI medium (10% heat inactivated fetal calf serum, 1X penicillin-streptomycin, 1X glutamine) at $5x10^6$ viable cells/ml, plated in a sterile 24-well tissue culture plate (1 ml/well) and cultured for 4 days at 37 ± 1°C with 5% CO_2 (cell viability not monitored). Supernatants were collected and frozen at -80 ± 10°C until assayed by ELISA.

α4β7 PBMC Separation. Frozen PBMCs from study days -1, 5 and 7 were thawed, washed and suspended at $1x10^7$ cells/ml in complete RPMI medium prior to incubation

with an Alexa Fluor 647 (AF647) conjugated anti-α4β7 monoclonal antibody (Act-1; NIH AIDS Reagent Program) for 10 minutes at 4 ± 2°C, protected from light. After washing, the cells were incubated with anti-AF647 MicroBeads (Miltenyi) for 15 minutes at 4 ± 2°C, protected from light. PBMCs were then washed and passed through a 30 µm cell strainer prior to separation of α4β7 positive and negative PBMC populations using a Miltenyi AutoMACS™. Both the α4β7+ and α4β7- populations were adjusted to $5x10^6$ cells/ml and cultured as described above to collect α4β7+ and α4β7- ALS. Immediately prior to ALS culture, 100 µl of both the α4β7+ and α4β7- populations was removed to assess population purity by flow cytometry. As α4β7+ cells were bound by the AF647 conjugated anti-α4β7 monoclonal antibody, it is expected that only the positive population will fluoresce when analyzed by flow cytometry. The α4β7+ and α4β7- populations for a given subject/time point were analyzed using a FACSCanto II and determined to be ≥90% pure post-separation (data not shown).

Memory B Cell Expansion and Quality Control. Frozen PBMCs from study days -1, 28 and 56 were thawed, washed and expanded as previously described[173] with minor modifications. Briefly, cells were suspended at $1x10^6$ cells/ml in complete RPMI medium with 2-mercaptoethanol and polyclonal mitogens: Pokeweed Mitogen extract at a 1:20,000 dilution (Sigma), CpG-ODN-2006 at 6 µg/ml (Invivogen) and fixed *Staphylococcus aureus* Cowan at a 1:10,000 dilution (Sigma). Cells were plated in a sterile 6-well tissue culture plate (3-5 ml/well) and cultured for 6 days at 37 ± 1˚C with 5% CO_2. After expansion, the cells were washed twice with mitogen-free complete RPMI medium, adjusted to $5x10^6$ cells/ml and cultured as described above to collect memory B cell ALS.

A novel method was developed to characterize the memory B cell expansion and establish acceptability criteria in order to objectively determine successful expansion of memory B cell populations (manuscript in preparation). Briefly, samples were analyzed by flow cytometry before and after polyclonal mitogen stimulation using T and B cell-specific markers (CD3-BV711, CD19-BV421, CD27-APC and CD20-PE-Cy7; BD Biosciences) and analyzed by flow cytometry using a FACSCanto II. Acceptance criteria for a successful expansion included assessing cell viability and cell concentrations pre- and post-expansion as well as ensuring an overall increase post-expansion in the percent of cells positive for the CD19 B cell marker. Additionally, an expansion was considered successful if B cell populations post-expansion showed a ≥20% increase in B cells positive for the CD27 memory marker, as compared to pre-expansion B cell populations.

Enzyme-Linked Immunosorbent Assay. Serum, stool extracts, and ALS samples were assayed by ELISA to determine *Shigella* antigen-specific antibody endpoint titers as previously described,[174] with the exception of the use of Immulon 1-B ELISA plates (Thermo) and human-specific secondary antibodies (reserve AP-conjugated Goat-Anti-Human IgG, IgA or IgM; Seracare; AP-conjugated Mouse-Anti-Human IgG1, IgG2, IgG3 or IgG4; Southern Biotech). ELISA titers were defined as the reciprocal of the last dilution of serum with an optical density (OD) above the assay cut-off value. Samples negative at the starting dilution were assigned a titer corresponding to half the starting dilution (½ LOD). Immune responders were defined a priori as having a ≥4-fold increase over their baseline titer.

Serum Bactericidal Activity (SBA). Antibody functionality was assessed by determining *Shigella*-specific SBA as previously described.[175] Briefly, serum samples, bacterial suspensions and 20% baby rabbit complement (BRC) were added to duplicate wells of a 96-well round-bottom plate (Corning). Assay controls included wells with bacteria and BRC only, and wells with bacteria and heat-inactivated BRC only. Plates were incubated at 37°C for 2 hours and subsequently placed on ice for 10 minutes. Samples were spot plated (10 µl/sample) onto square LB agar plates (Greiner Bio-One) and plates were tilted to allow the suspension to run down the agar (~2 cm) in order to distribute the bacterial colonies. Plates were allowed to dry, and incubated overnight at 37°C. The following day, a 2,3,5-triphenyltetrazolium chloride (TTC) agar overlay was added to the plates. As TTC is a cellular metabolism-indicating dye which stains bacterial colonies red, it allows bacterial colonies to be visualized and subsequently counted.

Colonies were enumerated using NICE software, and SBA titers were interpolated from a standard curve generated from the dilution series used in the assay, as previously described.[175,176] SBA titers were defined as the reciprocal serum dilution killing at least 50% of bacteria as compared to control wells. Serum samples were assayed starting at a 1:40 dilution and a titer of 20, corresponding to ½ LOD, was assigned to samples not exhibiting detectable serum bactericidal activity at the starting dilution. Immune responders were defined a priori as those with a ≥4-fold increase in SBA titer over baseline. Bactericidal activity directed to *S. sonnei*, Moseley, was determined, as well as to *S. flexneri* 2a, 2457T to investigate the cross reactivity of functional antibodies with other *Shigella* serotypes.

Disease Outcomes and Definitions. Immune responses pre- and post-challenge were compared across shigellosis outcome to determine the association of these immune parameters with progression to and severity of disease post-challenge. The outcome of shigellosis was chosen as at it is often the focus of *Shigella* vaccine development. However, immune responses demonstrating differences across additional disease outcomes are also presented. Diarrhea severity, dysentery (Appendix A) and disease severity score[105] were defined as previously described. While the primary clinical shigellosis endpoint was defined *a priori* in the clinical protocol and is used to describe attack rates in Appendix A, all immunological analyses were conducted using an alternative definition of shigellosis developed through a convening of *Shigella* CHIM experts focused on the standardization of *Shigella* CHIM clinical endpoints.[166] All disease outcomes used for immunological analyses are also described in Table 3.1.

Statistical Analyses. Normally distributed continuous immune response data were analyzed using appropriate parametric tests (T-Test or ANOVA) with Bonferroni post-hoc analyses as applicable. Non-normally distributed data were log-transformed prior to analysis in parametric tests, or appropriate non-parametric tests were used (Mann-Whitney U). A multivariate logistic regression model was developed to assess the association between various immune parameters at baseline and the odds of progressing to either shigellosis MSD versus none or mild diarrhea. Continuous variables were analyzed for assumption of linearity by plotting the variable against the log odds of the outcome. As age did not demonstrate a linear relationship with either outcome, it was appropriately categorized (MSD: 18-25, 26-40 and 41-49; shigellosis: 18-25 and 26-49).

Covariates of interest (gender, age category, race and log-transformed dose) were investigated in univariate models (α=0.20) and included in the final model if they were significantly associated ($p \leq 0.05$) with the outcome of interest, or influenced the effect estimate of baseline immune responses by ≥10% (gender, age category and log-transformed dose). Race did not meet either inclusion criteria and was excluded from the multivariate model. Cut-point analyses were performed by plotting receiver operating characteristics (ROC) and investigating sensitivity and specificity across different areas under the curve. Additional cut-point analyses were performed using Liu and Youden cut-point methods. All statistical tests were interpreted in a two-tailed fashion (α=0.05) with p-values ≤0.05 considered statistically significant in either Stata (Version 14 for MAC) or Prism (Version 7 for MAC).

3.5. RESULTS

Fecal Inflammatory Marker Responses. Fecal calprotectin and myeloperoxidase concentrations were determined only in stool samples from subjects consenting the use of their stool samples in future investigations (45/56 subjects). Of these 45 subjects, 18 progressed to shigellosis post-challenge while 26 did not. A substantial increase in calprotectin and myeloperoxidase (all $p \leq 0.002$, 2-way ANOVA) concentrations was observed 3 days post-challenge, regardless of shigellosis outcome, with concentrations returning to baseline levels by day 14 (Figures 3.1A and 3.1C). Subjects with shigellosis had significantly higher concentrations of both fecal inflammatory markers whether comparing day 3 concentrations (all $p \leq 0.018$, 2-way ANOVA; Figures 3.1A and 3.1C) or peak (observed on day 3 or 7) concentrations ($p=0.0002$ (calprotectin), $p=0.008$ (myeloperoxidase), T-test; Figures 3.1B and 3.1D) demonstrating an association of increased intestinal inflammation with progression to shigellosis. In addition, fecal inflammatory marker peak concentrations were associated with multiple other disease outcomes, including diarrhea severity, disease severity score and dysentery (Figures 3.2 and 3.3).

Serum Antibody Responses. *Shigella* antigen-specific serum IgG, IgA, IgM and IgG subclass responses were determined for all subjects and compared across groups with or without shigellosis. While baseline antigen-specific serum antibody levels were consistent with observations from previous *Shigella* CHIMs, additional investigations into baseline serum antibody levels are described in more detail below. LPS-specific serum IgG, IgA and IgM peaked 14 days post-challenge and remained elevated over baseline

levels 14- and 28-days post-challenge (all p≤0.003, 2-way ANOVA; Figure 3.4). There were no significant differences in LPS-specific serum IgG or IgA titers post-challenge between subjects with or without shigellosis (Figures 3.4A and 3.4B). LPS-specific serum IgM levels on day 14 were higher (p=0.033, 2-way ANOVA) in subjects with shigellosis (Figure 3.4C).

Serum IgG and IgA responses directed to additional antigens, including native invasion complex (Invaplex; IVP)[174] and IpaB and IpaC were investigated; however, no differences in peak serum titers post-infection were observed across shigellosis outcome groups (Table 3.2). IVP-specific titers showed the highest magnitude of response followed by IpaB and IpaC. Although a moderate increase (p=0.0008, 1-way ANOVA) in LPS-specific serum IgG1 titers was observed by day 14 across all subjects, there was no difference in peak LPS-specific serum IgG1 responses across subjects with or without shigellosis (Table 3.2). There were minimal to undetectable increases in LPS-specific serum IgG2, IgG3 and IgG4 responses post-infection, and no differences in peak titer in these IgG subclass responses across shigellosis outcome (Table 3.2).

α4β7 ALS Antibody Responses. PBMCs were collected prior to challenge (day -1) as well as 5 and 7 days post-challenge in an effort to capture the peak of lymphocyte circulation within in the blood. Robust LPS- and IVP-specific ALS IgA (all p≤0.0001, 2-way ANOVA; Figures 3.5A and 3.5C), and ALS IgG (all p≤0.0006, 2-way ANOVA; Figures 3.5B and 3.5D) responses from the α4β7+ B cells were observed 7 days post-infection, regardless of shigellosis outcome. Subjects with shigellosis had significant antigen-specific α4β7+ ALS IgA and IgG increases over baseline by day 5 post-infection across all antigens and isotypes (all p≤0.048, 2-way ANOVA; Figure 3.5). Although lower in

magnitude, similar trends in the α4β7- ALS IgA and IgG responses were observed with peak responses 7 days post-infection. With the exception of the LPS-specific α4β7- ALS IgA responses, subjects with shigellosis had higher LPS-specific α4β7- ALS IgG and IVP-specific α4β7- ALS IgG and IgA titers on day 7 as compared to baseline (all $p \leq 0.006$, 2-way ANOVA; Figures 3.5B, 3.5C and 3.5D).

Across both shigellosis outcome groups, day 7 LPS- and IVP-specific α4β7+ ALS IgA and IgG responses were higher than α4β7- ALS responses (all $p<0.0001$, 2-way ANOVA; Figure 3.5). LPS-specific α4β7+ ALS IgG and IVP-specific α4β7+ ALS IgA responses were also increased as compared to α4β7- ALS responses 5 days post-challenge ($p=0.037$ and $p=0.033$, respectively, 2-way ANOVA; Figures 3.5B and 3.5C).

Subjects progressing to shigellosis had substantially higher α4β7+ ALS responses on day 7 as compared to day 7 α4β7+ ALS responses in subjects without shigellosis (all $p<0.0001$, 2-way ANOVA; Figure 3.5). Interestingly, IVP-specific α4β7- ALS IgA and IgG responses on day 7 were significantly increased ($p=0.011$ and $p<0.0001$, respectively, 2-way ANOVA; Figures 3.5C and 3.5D) in subjects with shigellosis yet this same difference was not observed in the LPS-specific α4β7- ALS responses (Figures 3.5A and 3.5B). In addition to the association with shigellosis, LPS- and IVP-specific α4β7+ ALS IgA responses were associated with diarrhea severity, disease severity score and dysentery (Figures 3.6 and 3.7). LPS- and IVP-specific α4β7+ ALS IgG responses showed similar trends and were also associated with all aforementioned disease outcomes at similar levels of significance (data not shown).

Fecal Antibody Responses. Fecal IgA and IgG responses were determined for all subjects and compared across shigellosis outcome groups. In subjects with shigellosis, peak LPS-specific fecal IgA and IgG titers were observed 7 days post-infection, and although lower by day 14, remained elevated over baseline (all $p<0.0001$, 2-way ANOVA; Figures 3.8A and 3.8B). In contrast, LPS-specific fecal IgA and IgG responses in subjects without shigellosis continued to increase through study day 14 (all $p\leq0.049$, 2-way ANOVA; Figures 3.8A and 3.8B). LPS-specific fecal IgA and IgG titers on day 7 were higher in subjects with shigellosis as compared to subjects without (all $p\leq0.045$, 2-way ANOVA; Figures 3.8A and 3.8B) with similar trends and levels of significance observed in the IVP-specific fecal antibody responses (data not shown).

Fecal antibody responses were compared with α4β7+ ALS antibody responses, revealing a moderate correlation when peak fold-rise in fecal IgA or IgG was compared with peak fold-rise in α4β7+ ALS IgA or IgG ($r=0.51$ (IgA), $r=0.53$ (IgG), $p<0.0001$ Spearman correlation; Figures 3.8C and 3.8D).

Memory B Cell Responses. LPS- and IVP-specific memory B cell IgG and IgA ALS titers on day 28 were elevated over baseline (all $p\leq0.013$, 2-way ANOVA) and although responses decreased by day 56, ALS titers remained elevated over baseline levels (data not shown). The maximum fold-rise (day 28 or 56) over baseline was used to compare memory B cells responses across subjects with or without shigellosis post-challenge (Figure 3.9). With the exception of the LPS-specific memory B cell IgG responses, subjects with shigellosis had larger peak fold-rises in LPS-specific IgA and IVP-specific IgG and IgA

memory B cell ALS titers compared to subjects without shigellosis (all p≤0.027, Mann-Whitney U; Figure 3.9).

SBA Responses. Peak *S. sonnei*-specific SBA was observed 14 days post-challenge; however, levels were increased over baseline by day 7 (all p<0.0001, 2-way ANOVA; Figure 3.10A) and remained elevated through study day 56 (all p≤0.024, 2-way ANOVA; Figure 3.10A), regardless of shigellosis outcome. There were no differences in peak *S. sonnei*-specific bactericidal activity across subjects with or without shigellosis (Figure 3.10B) and although not significant, it is interesting to note that subjects without shigellosis had higher *S. sonnei*-specific bactericidal titers 28- and 56-days post-infection (Figure 3.10A).

Minimal increases in *S. flexneri* 2a-specific SBA over baseline were observed and there were no differences in peak titer (data not shown) or peak fold-rise over baseline (Figure 3.10C) across shigellosis outcome groups. A total of 8 subjects had a 4-fold or greater rise over baseline in *S. flexneri* 2a-specific bactericidal titers and interestingly, of these 8 subjects, 7 did not progress to shigellosis (Figure 3.10C).

Baseline Immune Responses. A thorough evaluation of pre-existing immunity was conducted to better understand the lack of association between disease severity and certain immune parameters (Figure 3.11) in this study population. As described in Appendix A, 171 subjects were screened for study inclusion. After exclusions based on other criteria, 130 subjects were screened for *S. sonnei* LPS-specific serum IgG titers between study days -45 to -2. Of these 130, 4 subjects (~3%) had titers greater than the 2500 cut-off used for study inclusion and were excluded from the study (Table 3.3).

Of the enrolled subjects, there were no differences in the LPS-specific serum IgG titers at baseline (study day -1) across shigellosis outcome group, and with the exception of one individual, all subjects had baseline LPS-specific serum IgG titers within 2-fold of the 2500 cut-off for inclusion (Figure 3.11A). In contrast, increased LPS-specific serum IgA baseline titers were associated with subjects not progressing to shigellosis (p=0.027, T-test; Figure 3.11A). A similar association was observed with baseline *S. sonnei*-specific SBA (p=0.038, Mann-Whitney U; Figure 3.11B) as well as LPS-specific fecal IgA and IgG titers at baseline (p=0.027 and p=0.035 respectively, Mann-Whitney U; Figure 3.11C). There was also a significant association of increased LPS-specific memory B IgA baseline titers with subjects not progressing to shigellosis (p=0.034, T-test; Figure 3.11D); however, this same association was not observed in the LPS-specific memory B IgG baseline titers (p=0.082, T-test; data not shown). LPS-specific serum IgA baseline titers were also inversely associated with diarrhea severity, dysentery and disease severity score (Figure 3.12).

Logistic regression analyses were used to investigate the association of increasing baseline LPS-specific serum IgG or IgA ELISA titers with progression to either mild to severe diarrhea (MSD), or to shigellosis (Table 3.4). Neither the unadjusted nor adjusted models showed an association of increasing baseline LPS-specific serum IgG with either outcome of interest. In contrast, both the unadjusted and adjusted models showed a significant association between progression to shigellosis or MSD and increasing baseline LPS-specific serum IgA titers. In the adjusted model, each fold-increase in baseline LPS-specific serum IgA titer resulted in nearly a 40% reduction in odds of progression to

shigellosis (odds ratio (OR)=0.61, 95% confidence interval (CI)=0.40-0.94; Table 3.4) with an area under the ROC of 0.777. Similarly, increasing baseline LPS-specific serum IgA titers resulted in nearly a 50% reduction in odds of progression to MSD (OR=0.51, CI=0.32-0.80; Table 3.4) with an area under the ROC of 0.831. Additional cut-point analyses suggest an LPS-specific serum IgA titer of ≤300 or ≤150 as optimal cut-points for predicting progression to MSD or shigellosis, respectively (data not shown).

3.6. DISCUSSION

The primary objective of the *S. sonnei* 53G CHIM was to determine a dose of a cGMP manufactured lyophilized strain that would safely and reproducibly induce a ≥60% shigellosis rate in challenged subjects (Appendix A). The study also provided a unique opportunity to extensively characterize the *Shigella*-specific systemic, mucosal and functional antibody immune responses pre- and post-infection with *S. sonnei* 53G in a more robust manner than previous studies. The more expansive immune response characterization further facilitates the understanding of host resistance to infection and could lead to determining immune markers that may be associated with recovery from shigellosis and/or protection against illness upon re-exposure. As expected, infection with *S. sonnei* induced robust intestinal inflammatory and mucosal responses as observed by sharp increases in fecal inflammatory markers and α4β7+ ALS antibody responses in diseased subjects, and both of these immune parameters were significantly associated with multiple disease outcomes. Interestingly, there was a lack of association with post-infection systemic immune responses (serum antibodies and bactericidal activity) and disease outcomes. The relationship between intestinal inflammatory markers and disease outcomes noted above is intriguing and deserves further investigation into its potential relationship to immune outcomes that are associated with recovery from acute illness or may be predictive of a reduced risk of illness when re-exposed to *Shigella*.

Fecal IgA responses are often used as a measure of the mucosal immune response post-infection with *Shigella*. In this study, fecal IgG responses were also

evaluated. Antibody responses from B cells positive for the gut homing marker α4β7 were also determined and presented as a measure of the mucosal immune response. As α4β7+ cells home to the intestinal mucosa and secrete IgA and IgG antibodies that are actively transported or passively transudated into the lumen of the large intestine, the correlation of these two mucosal measurements was of particular interest. The observed correlation of α4β7+ ALS IgG and fecal IgG suggests that the IgG secreting α4β7+ cells may be partially responsible for the antigen-specific IgG detected in stool samples in addition to IgG transudate from systemic circulation.

Antecedent shigellosis reduces the risk of subsequent infection in a serotype-specific manner, indicating a role for LPS-specific adaptive immune responses in protection.[98,99,170,177] LPS-specific serum IgG has been implicated as an immune correlate of protection responsible for this observed serotype-specific protection;[114,122] however, several of these studies have investigated serum IgG levels after parenteral immunization with conjugate vaccines which would be expected to induce high amounts of LPS-specific serum IgG.[116,122,178-180] Furthermore, immunological analyses in many previous studies have been limited with incomplete analysis of other immune parameters outside of serum IgG. In contrast, results from this report and other studies evaluating mucosal routes of immunization or infection[106,114,181,182] suggest that LPS-specific IgA also contributes to protection. Together, these data provide insight that there exist alternate mechanisms to induce a protective immune response, depending on the route of antigen exposure.

Interestingly, increased LPS-specific serum IgA, IgA-secreting memory B cells and SBA titers at baseline were inversely associated with risk of shigellosis. Increased baseline serum IgA titers have also been associated with reduced risk of disease in enterotoxigenic *Escherichia coli* (ETEC) CHIMs[183] as well as reduced susceptibility to cholera infection in endemic settings.[182] In an effort to recruit immunologically naïve subjects, exclusion criteria for this study included recent travel to *Shigella* endemic areas, known history of culture confirmed *Shigella* infection and a *S. sonnei* LPS-specific serum IgG ELISA titer of >2500, however, LPS-specific serum IgA responses were not assessed during study screening. The serum IgG exclusion criterion used in this study was developed using data from a prior *Shigella* CHIM demonstrating that subjects with disease post-oral challenge had a serum IgG ELISA titer of at least 2500.[169] While this serological exclusion criterion has been applied to several *Shigella* vaccine studies and CHIMs,[127,184,185] results described here suggest it may be insufficient, even when combined with exclusion based on previous *Shigella* exposure and travel.

As outlined above, memory B cell IgA responses directed to LPS correlated with resistance to shigellosis. In addition, LPS-specific serum IgA responses and SBA also were associated with decreased risk of shigellosis, albeit at a lower magnitude. These results demonstrate that immune parameters other than serum IgG may contribute to resistance to shigellosis. Given these associations with resistance to shigellosis, it may be reasonable to expand the immunological parameters utilized for excluding subjects from *Shigella* vaccine studies or CHIMs when a naïve population is critical. While data presented here suggests that LPS-specific IgA-secreting memory B cell responses at

baseline may be the most suitable predictor of resistance to shigellosis, a balance needs to be maintained between subject exclusion criteria and the ability to successfully recruit subjects. Therefore, impractical, costly, or technically difficult screening assays must be weighed against study objectives. Functional immune responses at baseline, as determined by SBA, could also be considered; however, the association of baseline SBA with disease outcomes was less robust compared to LPS-specific serum IgA responses and the assay is also more technically challenging and time-consuming as compared to an ELISA. Collectively, these results suggest that LPS-specific serum IgA titers should be considered as a potentially useful and practical tool to more reliably exclude subjects with pre-existing immunity to *Shigella*, similar to ETEC CHIMs.[101] Although setting additional or more stringent exclusion criteria may become problematic when developing CHIMs in LMICs, an alternative strategy may be to stratify and/or randomize subjects based on baseline immunity.

Logistic regression analyses indicated an approximate 40% and 50% reduction in odds of progression to shigellosis and MSD, respectively; however, these same associations were not observed with baseline LPS-specific serum IgG titers. This lack of association with baseline LPS-specific serum IgG and progression to shigellosis or MSD may be due to the fact that subjects with high baseline serum IgG titers were excluded from the study, potentially providing a biased population after study enrollment. However, of the 130 subjects that underwent LPS-specific serum IgG screening, only 4 subjects (3.1%) were excluded based on high *S. sonnei* LPS-specific serum IgG titers

(Table 3.3). Additionally, of the 56 enrolled subjects, 41 (73%) had a LPS-specific serum IgG screening titer of ≤625.

ROC analyses revealed an optimal baseline LPS-specific serum IgA cut-point of ≤300 for predicting progression to MSD. In an effort to investigate the lack of a dose response observed in this CHIM (Appendix A), systemic immune responses were re-analyzed by approximate *Shigella* dose (500, 1000 or 1500 cfu) excluding subjects with a baseline LPS-specific serum IgA titer >400; however, removal of these subjects did not result in a dose-dependent LPS-specific serum IgG response or SBA response. This lack of association could be a function of the sample size (N=40) resulting in insufficient power to determine significant differences, or it could imply that there are other host factors, such as microbiome or genetics, that have not yet been measured or explored.

There are multiple immune mechanisms that could be employed to protect against *Shigella*, which can be effective during the intracellular and extracellular phases of the bacterial infection. Protective immune responses could be driven, in part, by antibodies in the lumen preventing transcytosis of the bacteria across the epithelial cell layer (whether secretory IgA or IgG transudate), complement activation in the lamina propria or antibody dependent cellular cytotoxicity once *Shigella* has reached the intracellular phase of its life cycle. While assessing mechanistic correlates of protection is important, investigating immunological surrogates that represent the mechanistic correlates of protection is also informative. Additionally, rather than identifying one immune parameter responsible for protection, there may be multiple immune parameters working in concert, effectively defining a protective immune profile.[168]

Furthermore, depending on the initial route of antigenic exposure, there may be distinct protective immune profiles generated. Additional analyses, including *Shigella*-specific salivary antibody responses, determination of Fc glycosylation patterns, *Shigella* microarray analyses, transcriptomics and systems serology are currently planned to parse out more nuanced differences in immune responses across the disease spectrum. Similarly, ongoing analyses and determination of a protective immune profile in the context of parenterally administered *Shigella* vaccines could offer additional insights.

Disease outcomes and immune responses post-infection may also be affected by the duration of antigenic exposure. In the *S. sonnei* 53G CHIM, the majority of subjects were administered antibiotics 5 days post-inoculation (or sooner if clinically indicated, N=9) which often does not occur during natural infection. Interrupting the infection with antibiotics may reduce the amount and exposure time to *Shigella* antigens potentially impacting the magnitude, specificity, duration, maturation and phenotype of the immune response induced in a CHIM,[186] as compared to endemic settings. Depending on the duration of antigenic stimulation, more robust or perhaps different immune responses could occur, including generation of antibodies with higher affinity or avidity, antibodies with different Fc glycosylation patterns, or increased activation of cell-mediated immune mechanisms.

Additional immune response analyses by time and length of shedding *S. sonnei* in stool samples could also provide important insights into the relationship between *Shigella* infection and immune responses. Unfortunately, the current study was not powered to detect differences in immune responses across culture confirmed *S. sonnei*

shedding as the majority of subjects had culture negative stools within 3-5 days after their first culture-positive stool sample. Efforts are currently underway to quantify *S. sonnei* in stool samples using qPCR detection methods in order to further investigate the immune responses and *S. sonnei* shedding in stool samples post-infection.

Other non-immunological factors may also contribute to the resistance to infection and lack of association of some immune parameters with disease outcomes observed in this study. While a CHIM attempts to control for many variables, such as challenge dose and age, there remain several uncontrolled factors in both CHIMs and natural infection settings that could contribute to differences in disease outcomes. Population genetics, prior exposure, contemporaneous host immune status or co-infections, as well as microbiome and nutritional status may affect immune response profiles and disease outcomes post-infection.[131-134] Finally, this study was conducted in healthy, North American adults who are a distinctly different population than the target population of children <2 years old living in LMICs. Infant immune systems do not fully mature until approximately 24 months of age and have been shown to have lower levels of circulating immunoglobulin and complement effectors leading to different immune responses being required to induce protection in this population.[187] Furthermore, infant health and immune status can vary greatly in endemic settings, so caution is required when extrapolating immune responses post-infection in healthy North American adults.

Nonetheless, as conducting a *Shigella* CHIM in the target population of LMIC children is not possible, data collected in this study are invaluable to understanding immune responses and their association with disease post-challenge with *S. sonnei*. In

addition to the immune response characterization post-infection, this study provides important information about immune status pre-infection. Increased levels of serum IgA may be a contributing factor to resistance after oral *Shigella* challenge, and while additional investigations with increased group numbers is important, this study has been able to provide some guidance on immunological screening assays for use in future *Shigella* CHIMs.

Ideally, protective immunity against shigellosis would be assessed in the context of a challenge/re-challenge CHIM study design, however, the current study was designed and powered to only answer the primary clinical outcome. Nonetheless, as prior disease results in protective immunity against subsequent infection with the same *Shigella* serotype, subjects in this trial provide an important population for the investigation of potential immune correlates or surrogates of protection as those with moderate to severe disease would likely be protected against reinfection.[98,109,177] With the dose of *S. sonnei* 53G now established, next steps should focus on the conduct of a challenge/re-challenge study in order to fully elucidate the immunological mechanisms responsible for protection from shigellosis. Additionally, a heterologous challenge/re-challenge study is in the planning stages to investigate the potential of cross-protection provided across different *Shigella* species. The *S. sonnei* 53G CHIM has confirmed the relevance of robust mucosal immune responses post-infection and their potential role as a mechanistic correlate of protection in this model,[120] while suggesting that systemic immune responses may play a lesser role as they were not as reflective of disease outcome and severity in this study. The observations that baseline levels of LPS-specific serum IgA and

IgA-secreting memory B cells are associated with reduced odds of disease point to these immune measures as being potentially more sensitive markers for underlaying protective immunity and therefore should be further studied. Together, these data may help guide the rational design of future *Shigella* vaccines while representing a framework for future studies and functioning as a benchmark for comparisons across CHIM.

Table 3.1. Disease Outcome Definitions and Source

Outcome	Definition	Scale
Diarrhea Severity (Chapter 3)	Diarrhea severity is divided into the following 4 categories: 1) None 2) Mild: 2-3 grade 3-5 stools and <400g of grade 3-5 stools in 24hrs 3) Moderate: 4-5 grade 3-5 stools or 400-800g of grade 3-5 stools in 24hrs 4) Severe: ≥6 grade 3-5 stools or >800g of grade 3-5 stools in 24hrs Source: Defined per protocol (R.W. Frenck *et al*. 2020, Manuscript Submitted)	**Binary Outcome:** Subjects were combined into 1 of 2 categories: 1) Those with no diarrhea or mild diarrhea 2) Those with moderate or severe diarrhea
Dysentery (Chapter 3)	A least 2 grade 3-5 stool with gross blood with reportable constitutional/enteric symptoms. Source: Defined per protocol (R.W. Frenck *et al*. 2020, Manuscript Submitted)	**Binary Outcome:** Subjects grouped by whether or not they received a dysentery diagnosis during the study
Disease Severity Score (Chapter 3)	Subjects are assigned scores (as described by Porter *et al*.) across the following categories depending on severity and frequency: 1) Objective Symptoms: gross blood, oral temperature, vomiting 2) Subjective Symptoms: constitutional/enteric symptom 3) Grade 3-5 stool output per 24hrs Source: Porter *et al*. 2018, DOI: 10.1371/ journal.pone.0194325	**Ordinal Outcome:** Subjects' scores across three categories are added up to for a final score between 0 – 9
Consensus Shigellosis (Chapter 3 & Chapter 4)	One of three definitions (as described by MacLennan *et al*.) must be met: 1) Severe diarrhea 2) Moderate diarrhea with one of the following: fever ≥38.0°C or ≥1 moderate constitutional/enteric symptom or ≥2 episodes of vomiting in 24hrs 3) Dysentery with one of the following: fever ≥38.0°C or ≥1 moderate constitutional/enteric symptom or ≥2 episodes of vomiting in 24hrs Source: MacLennan *et al*. 2019, DOI: 10.1093/cid/ciz891	**Binary Outcome:** Subjects grouped by whether or not they received a shigellosis diagnosis during the study
***S. flexneri* 2a Per Protocol Shigellosis** (Chapter 4)	One of three definitions (as described by Talaat *et al*.) must be met: 1) Severe diarrhea (as defined above) 2) Moderate diarrhea (as defined above) with one of the following: fever ≥38.0°C or ≥1 moderate constitutional/enteric symptom 3) Dysentery (as defined above) with ≥1 constitutional/enteric symptom Source: Defined per protocol (K.R. Talaat *et al*. 2020, Manuscript Submitted)	**Binary Outcome:** Subjects grouped by whether or not they received a shigellosis diagnosis during the study

Table 3.2. *Shigella* Antigen-Specific Serum Antibody Responses at Baseline (Day -1) and Peak Titer Post-Baseline (Day 14, 28 or 56) with Number and Percent Responders

	Disease Outcome				
	No Shigellosis (N = 34)		Shigellosis (N = 22)		
Immune Parameter	GMT[a]	Responders (%)[b]	GMT	Responders (%)	P-Value[c]
IVP-Specific Serum IgG					
Baseline	2,832	---	1,704	---	
Peak Titer	10,654	14/34 (41%)	11,646	14/22 (64%)	0.793
IVP-Specific Serum IgA					
Baseline	347	---	129	---	
Peak Titer	3,924	23/34 (68%)	6,400	22/22 (100%)	0.210
IpaB-Specific Serum IgG					
Baseline	521	---	171	---	
Peak Titer	3,767	21/34 (62%)	1,651	15/22 (68%)	0.179
IpaB-Specific Serum IgA					
Baseline	78	---	62	---	
Peak Titer	326	14/34 (41%)	234	8/22 (36%)	0.546
IpaC-Specific Serum IgG					
Baseline	326	---	353	---	
Peak Titer	511	4/34 (12%)	683	4/22 (18%)	0.470
IpaC-Specific Serum IgA					
Baseline	87	---	83	---	
Peak Titer	113	3/34 (9%)	107	2/22 (9%)	0.843
LPS-Specific Serum IgG1					
Baseline	55	---	55	---	
Peak Titer	153	12/34 (35%)	100	5/22 (23%)	0.253
LPS-Specific Serum IgG2					
Baseline	80	---	71	---	
Peak Titer	141	8/34 (24%)	113	5/22 (23%)	0.456
LPS-Specific Serum IgG3					
Baseline	50	---	52	---	
Peak Titer	69	6/34 (17%)	73	3/22 (14%)	0.785
LPS-Specific Serum IgG4					
Baseline	50	---	50	---	
Peak Titer	50	0/34 (0%)	50	0/22 (0%)	>0.999

[a] GMT = Geometric Mean Titer

[b] Responder defined as a subject with a ≥4-fold increase in titer over baseline.

[c] Significance comparing peak titer in subjects with or without shigellosis. P-value determined by T-test of log-transformed titers.

Table 3.3. *S. sonnei* LPS-Specific Serum IgG Screening ELISA Titers Across Screened Subjects, Enrolled Subjects and Subjects that Progressed to Shigellosis

LPS-Specific Serum IgG ELISA Titer	Total Number of Subjects					
	Screened (N = 130)[a]	Percent[b]	Enrolled (N = 56)	Percent[c]	Shigellosis (N = 22)	Percent[d]
Category 1: <625	66	51%	27	48%	13	59%
Category 2: 625	25	19%	14	25%	4	18%
Category 3: 1250	25	19%	10	18%	4	18%
Category 4: 2500	10	8%	5	9%	1	5%
Category 5: ≥5000	4	3%	N/A[e]	---	N/A	---

[a] Of the 171 subjects screened for study inclusion, a total of 130 subjects underwent serological screening.
[b] Calculated as (number of subjects within a given ELISA titer category / total number of subjects screened by ELISA)
[c] Calculated as (number of enrolled subjects within a given ELISA titer category / total number of enrolled subjects)
[d] Calculated as (number of subjects with shigellosis within a given ELISA titer category / total number of subjects with shigellosis)
[e] N/A = Not Applicable

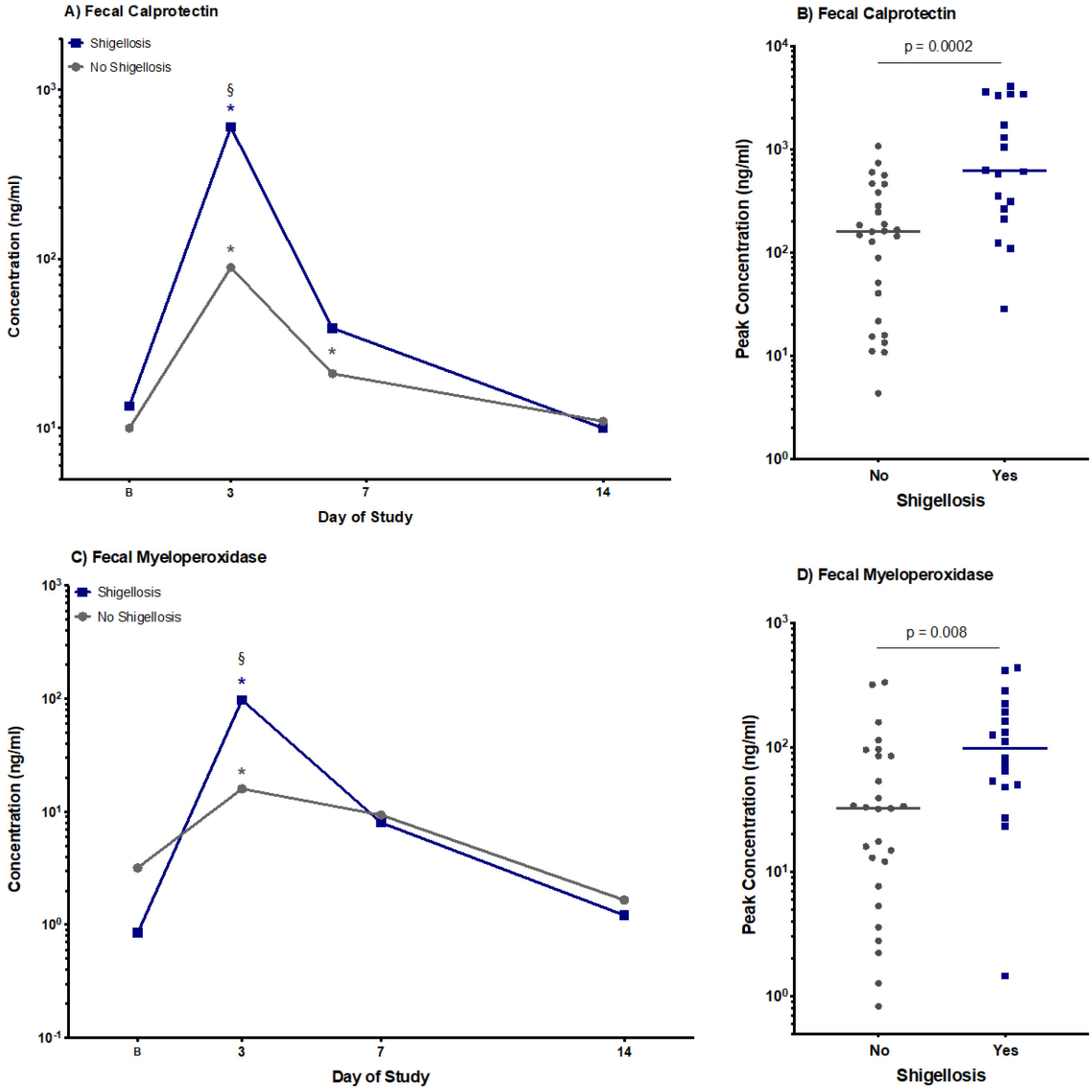
Table 3.4. Logistic Regression Models Investigating the Association of Progression to Shigellosis or Moderate-Severe Diarrhea with Baseline *S. sonnei* LPS-Specific Serum IgG and IgA ELISA Titers

Outcome of Interest		Odds Ratio (Confidence Interval)			
		Unadjusted OR[a]	P-Value	Adjusted OR[b]	P-Value
Shigellosis					
Gender:	Male (ref)	1.00		1.00	
	Female	0.55 (0.18 – 1.63)	0.277	0.37 (0.09 – 1.47)	0.158
Age:	18 – 25 (ref)	1.00		1.00	
	26 – 49	0.69 (0.20 – 2.42)	0.564	0.54 (0.11 – 2.60)	0.439
Dose (cfu)		**7.65 (1.44 – 40.6)**	**0.017**	**15.3 (1.91 – 122.0)**	**0.010**
Baseline LPS-Specific Serum IgG		0.89 (0.61 – 1.29)	0.534	1.27 (0.72 – 2.24)	0.404
Baseline LPS-Specific Serum IgA		**0.70 (0.51 – 0.97)**	**0.033**	**0.61 (0.40 – 0.94)**	**0.026**
Moderate-Severe Diarrhea					
Gender:	Male (ref)	1.00		1.00	
	Female	1.03 (0.35 – 2.98)	0.961	1.61 (0.40 – 6.53)	0.504
Age:	18 – 25 (ref)	1.00		1.00	
	26 – 40	1.39 (0.36 – 5.30)	0.633	1.40 (0.23 – 8.51)	0.712
	41 – 49	2.40 (0.52 – 11.0)	0.259	7.21 (0.87 – 59.6)	0.067
Dose (cfu)		**5.08 (1.10 – 23.5)**	**0.038**	**15.9 (1.95 – 129.5)**	**0.010**
Baseline LPS-Specific Serum IgG		0.79 (0.55 – 1.15)	0.216	1.18 (0.66 – 2.09)	0.582
Baseline LPS-Specific Serum IgA		**0.60 (0.43 – 0.85)**	**0.004**	**0.51 (0.32 – 0.80)**	**0.004**

[a] OR = Odds Ratio

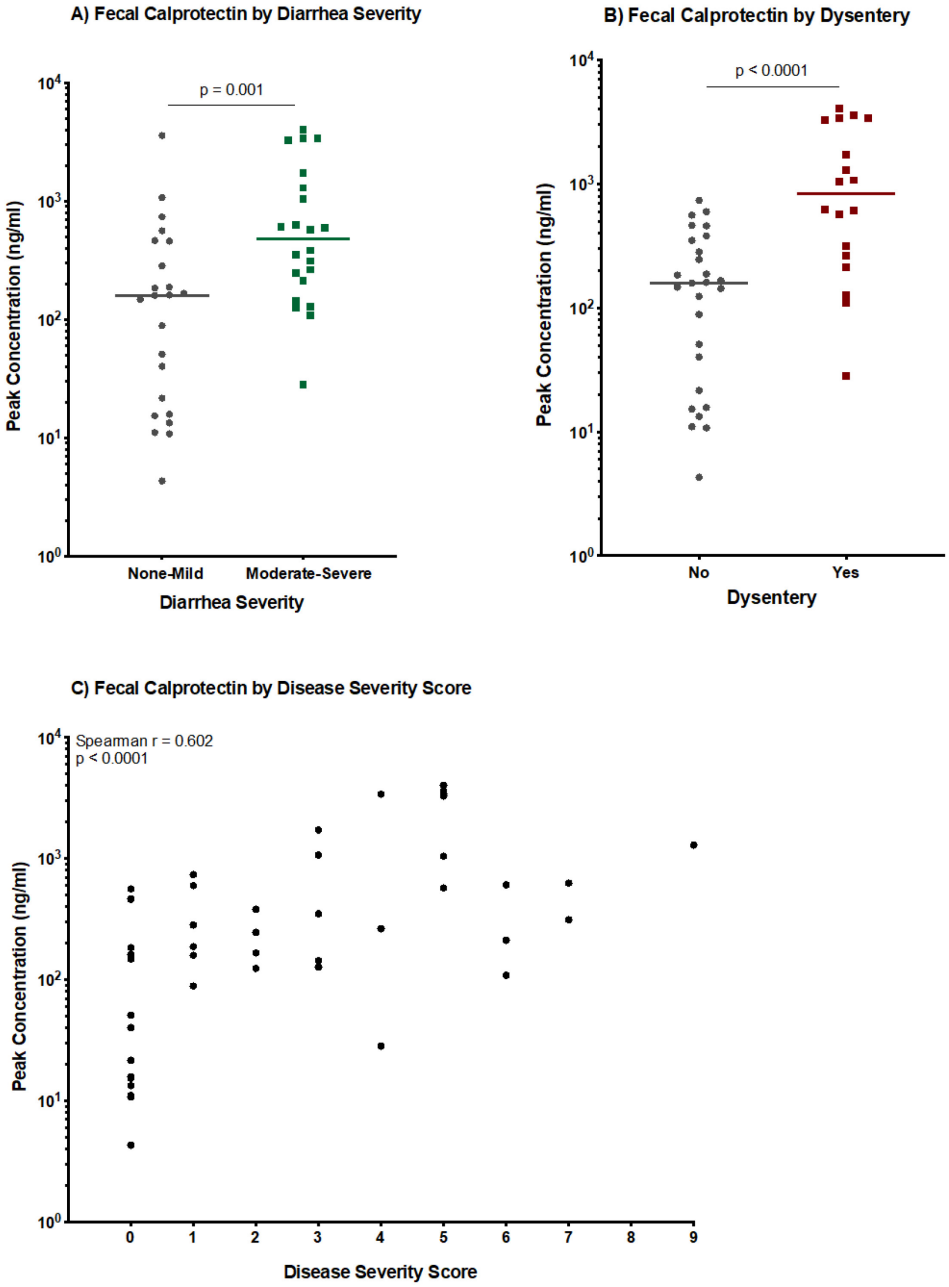
[b] Model adjusted for gender, age category, log-transformed dose and baseline LPS-specific serum IgG and IgA ELISA titer

Figure 3.1. Fecal Calprotectin and Myeloperoxidase Concentrations after Oral Challenge with *S. sonnei* 53G



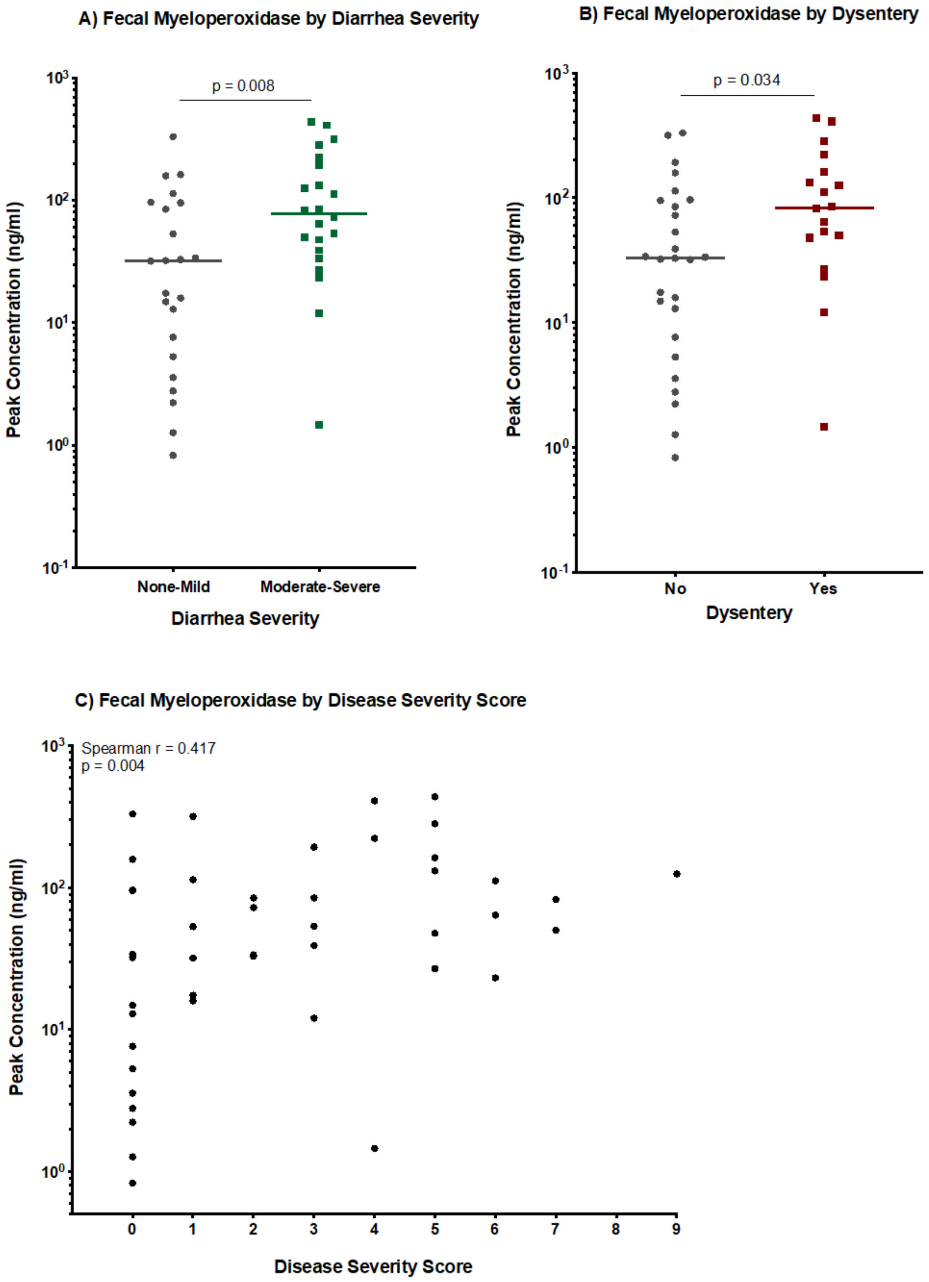
Median (A) fecal calprotectin and (C) myeloperoxidase concentrations at baseline/B and 3, 7 and 14 days post-challenge, grouped by subjects with (N=18) or without (N=27) shigellosis. * = significant difference as compared to baseline concentrations within shigellosis outcome group; § = significant difference in concentrations between shigellosis groups at the same time point. Significance determined by 2-way ANOVA of log-transformed concentrations with Bonferroni post-hoc test. Individual peak (B) fecal calprotectin and (D) myeloperoxidase concentrations with group median. P-value determined by T-test of log-transformed concentrations.

Figure 3.2. Fecal Calprotectin Concentrations after Oral Challenge with *S. sonnei* 53G by Additional Disease Outcomes



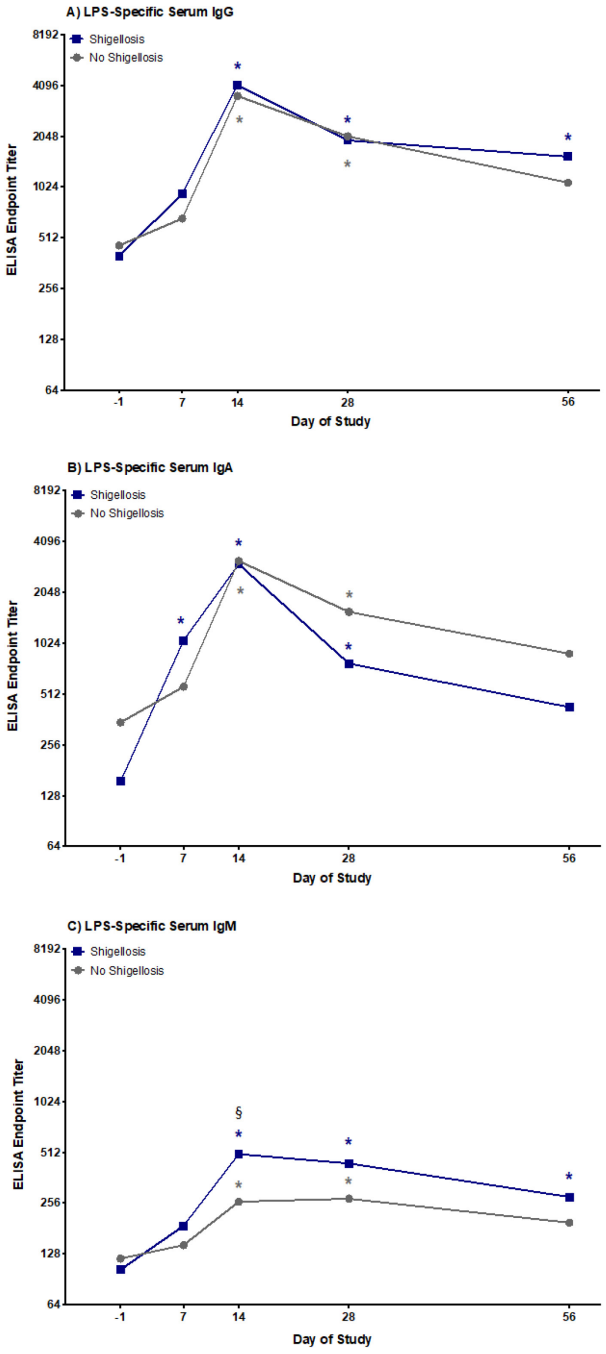
Individual peak fecal calprotectin concentrations with group median, grouped by (A) subjects with none-mild diarrhea (N=23) or moderate-severe diarrhea (N=22), and (B) subjects with (N=18) or without (N=27) dysentery. P-value determined by T-test of log-transformed concentrations. (C) Spearman correlation of individual peak fecal calprotectin concentrations and disease severity score (N=45).

Figure 3.3. Fecal Myeloperoxidase Concentrations after Oral Challenge with *S. sonnei* 53G by Additional Disease Outcomes



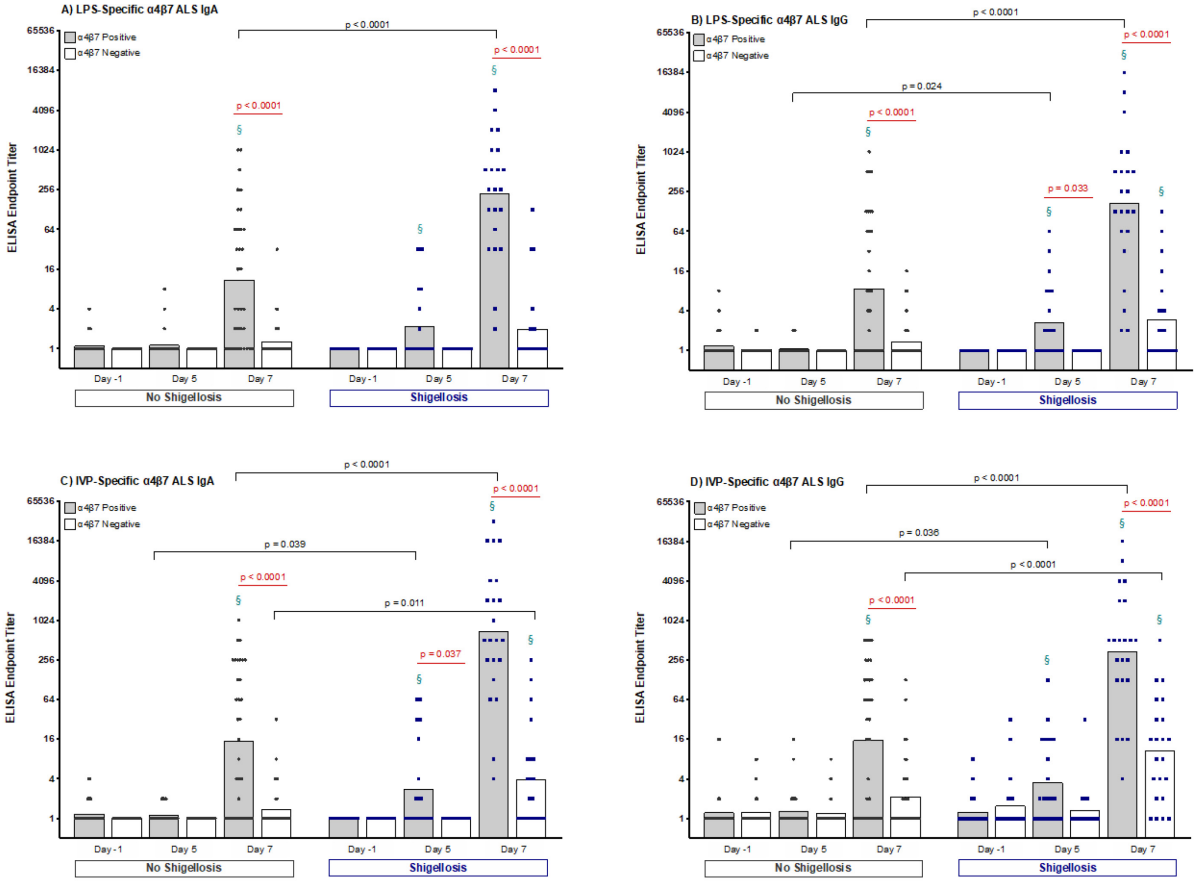
Individual peak fecal myeloperoxidase concentrations with group median, grouped by (A) subjects with none-mild diarrhea (N=23) or moderate-severe diarrhea (N=22), and (B) subjects with (N=18) or without (N=27) dysentery. P-value determined by T-test of log-transformed concentrations. (C) Spearman correlation of individual peak fecal myeloperoxidase concentrations and disease severity score (N=45).

Figure 3.4. *S. sonnei* LPS-Specific Serum IgG, IgA and IgM Responses



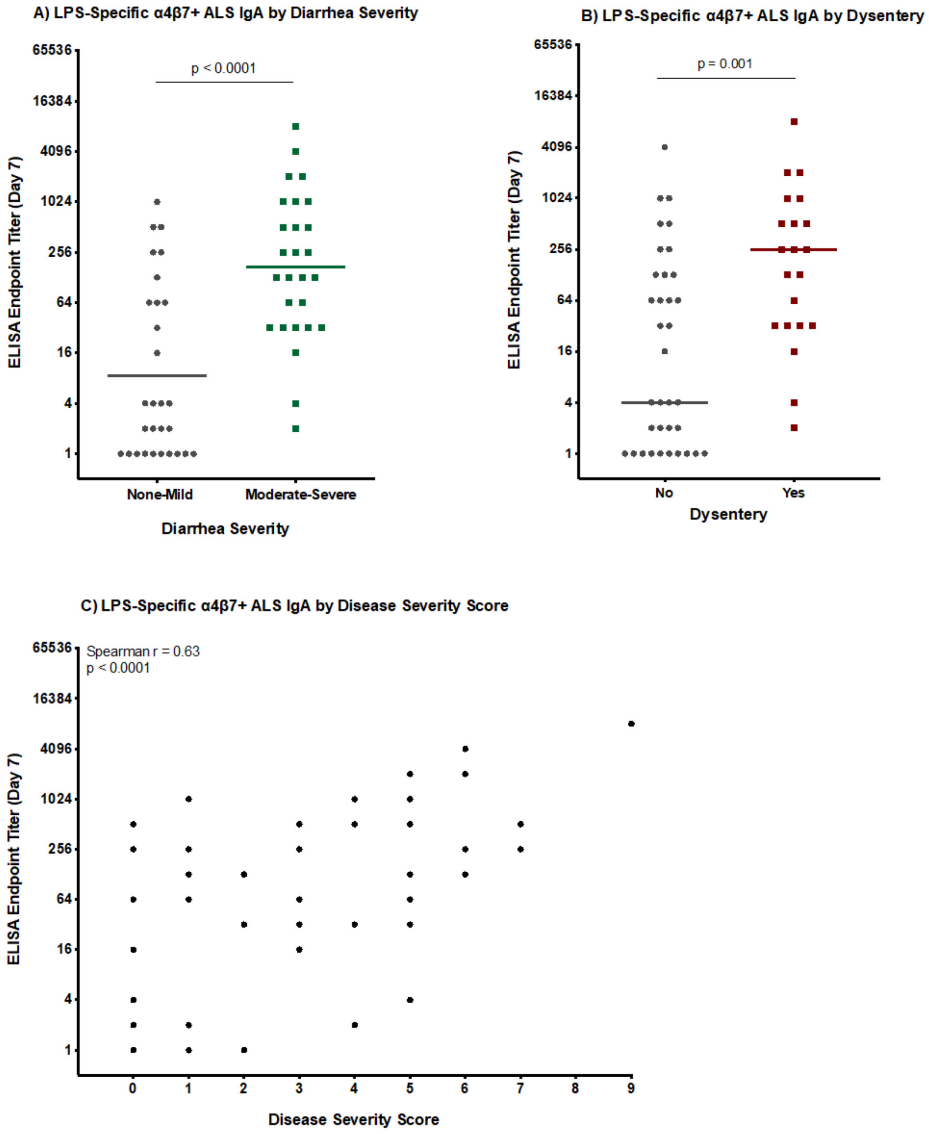
S. sonnei LPS-specific serum (A) IgG, (B) IgA and (C) IgM geometric mean ELISA endpoint titers prior to challenge (day -1) and 7, 14, 28 and 56 days post-challenge, grouped by subjects with (N=22) or without (N=34) shigellosis. * = significant difference as compared to baseline titers within shigellosis group; § = significant difference in titers between shigellosis groups at the same time point. Significance determined by 2-way ANOVA of log-transformed titers with Bonferroni post-hoc test.

Figure 3.5. *S. sonnei* LPS and Invaplex-Specific α4β7+ and α4β7- ALS IgG and IgA Responses



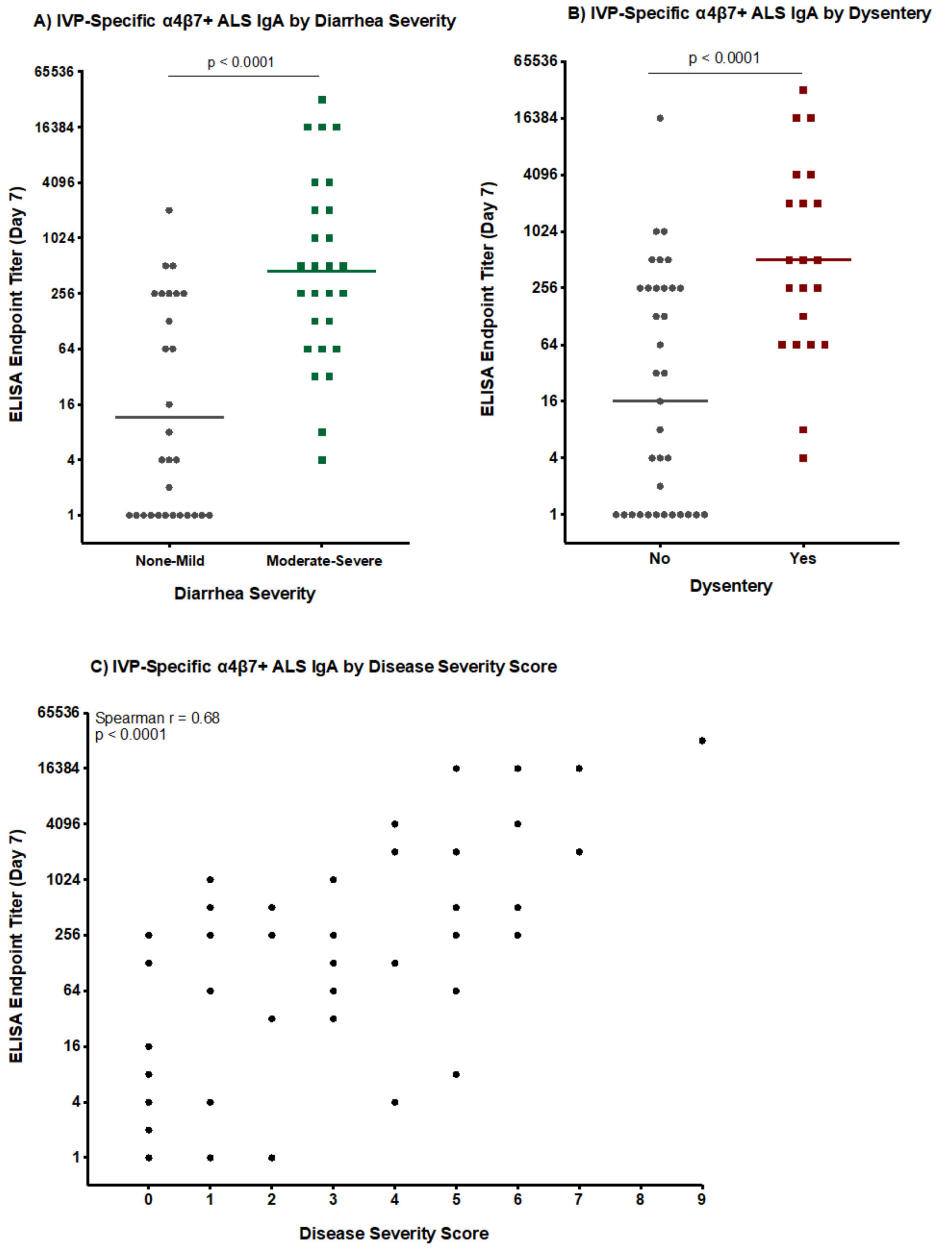
Individual ALS IgA and IgG ELISA endpoint titers with group geometric mean for α4β7+ (grey bars) and α4β7- (white bars) populations prior to challenge (day -1) and 5 & 7 days post-challenge, grouped by subjects with (N=22) or without (N=34) shigellosis. (A) *S. sonnei* LPS-specific ALS IgA; (B) *S. sonnei* LPS-specific ALS IgG; (C) *S. sonnei* IVP-specific ALS IgA; (D) *S. sonnei* IVP-specific ALS IgG. § = significant as compared to matched α4β7+ or α4β7- baseline ALS titers within shigellosis group. P-values in red compare α4β7+ and α4β7- ALS titers on a given study day within shigellosis group. P-values in black compare matched α4β7+ or α4β7- ALS titers on a given study day across shigellosis group. Significance across all parameters determined by 2-way ANOVA of log-transformed ALS titers with Bonferroni post-hoc test.

Figure 3.6. *S. sonnei* LPS-Specific α4β7+ ALS IgA Responses by Additional Disease Outcomes



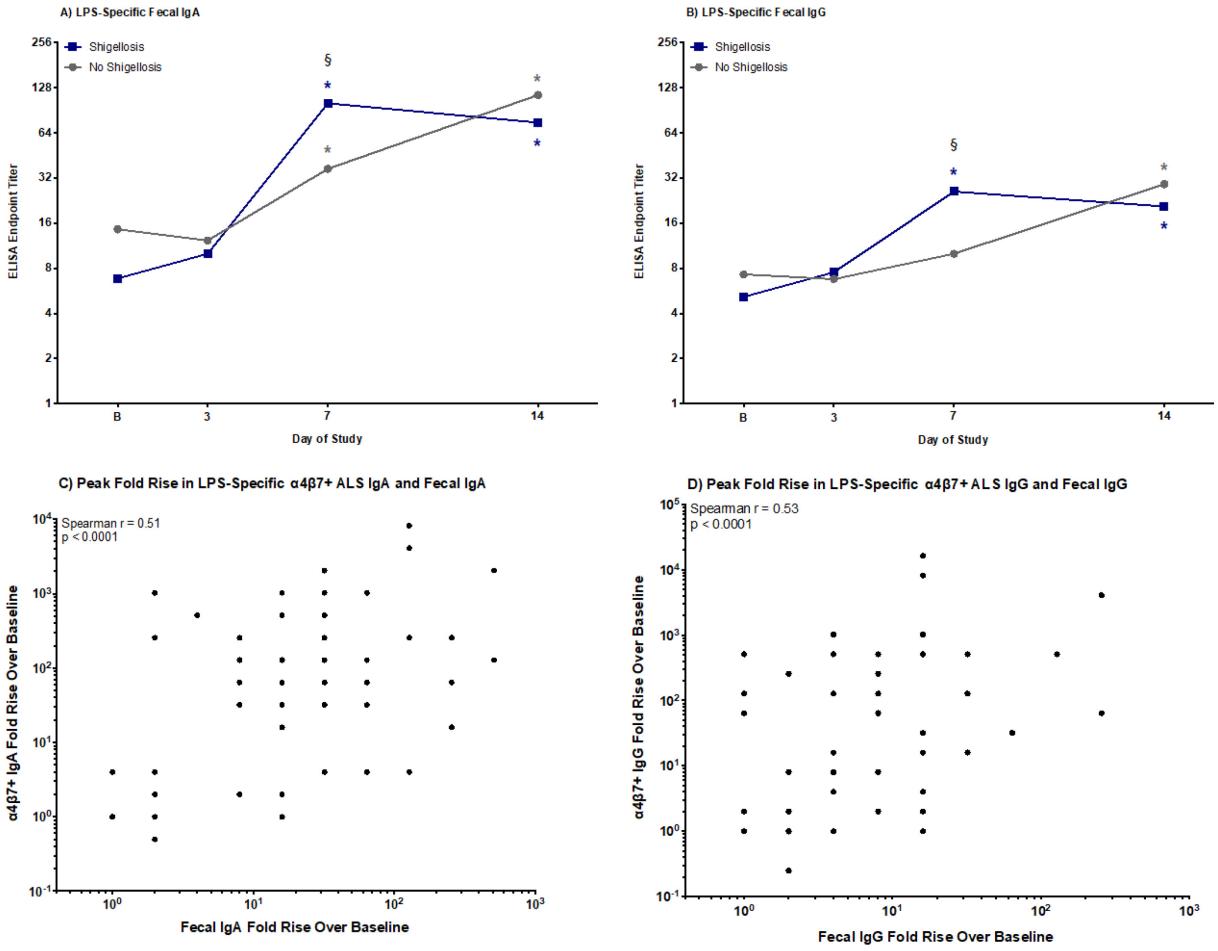
Individual day 7 *S. sonnei* LPS-specific α4β7+ ALS IgA ELISA endpoint titers with group geometric mean, grouped by (A) subjects with none-mild diarrhea (N=29) or moderate-severe diarrhea (N=27), and (B) subjects with (N=21) or without (N=35) dysentery. P-value determined by T-test of log-transformed titers. (C) Spearman correlation of day 7 *S. sonnei* LPS-specific α4β7+ ALS IgA ELISA endpoint titers and disease severity score (N=56).

Figure 3.7. *S. sonnei* Invaplex-Specific α4β7+ ALS IgA Responses by Additional Disease Outcomes



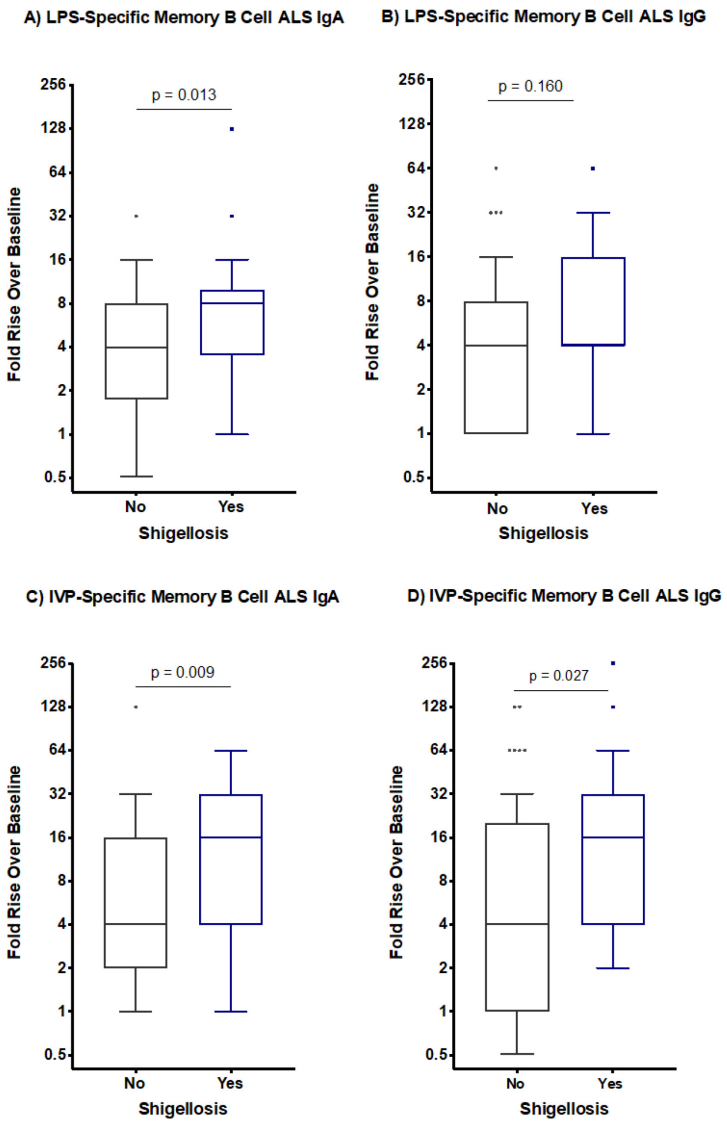
Individual day 7 *S. sonnei* IVP-specific α4β7+ ALS IgA ELISA endpoint titers with group geometric mean, grouped by (A) subjects with none-mild diarrhea (N=29) or moderate-severe diarrhea (N=27), and (B) subjects with (N=21) or without (N=35) dysentery. P-value determined by T-test of log-transformed titers. (C) Spearman correlation of day 7 *S. sonnei* IVP-specific α4β7+ ALS IgA ELISA endpoint titers and disease severity score (N=56).

Figure 3.8. *S. sonnei* LPS-Specific Fecal IgA and IgG Responses and their Correlation with α4β7+ ALS IgA and IgG Responses



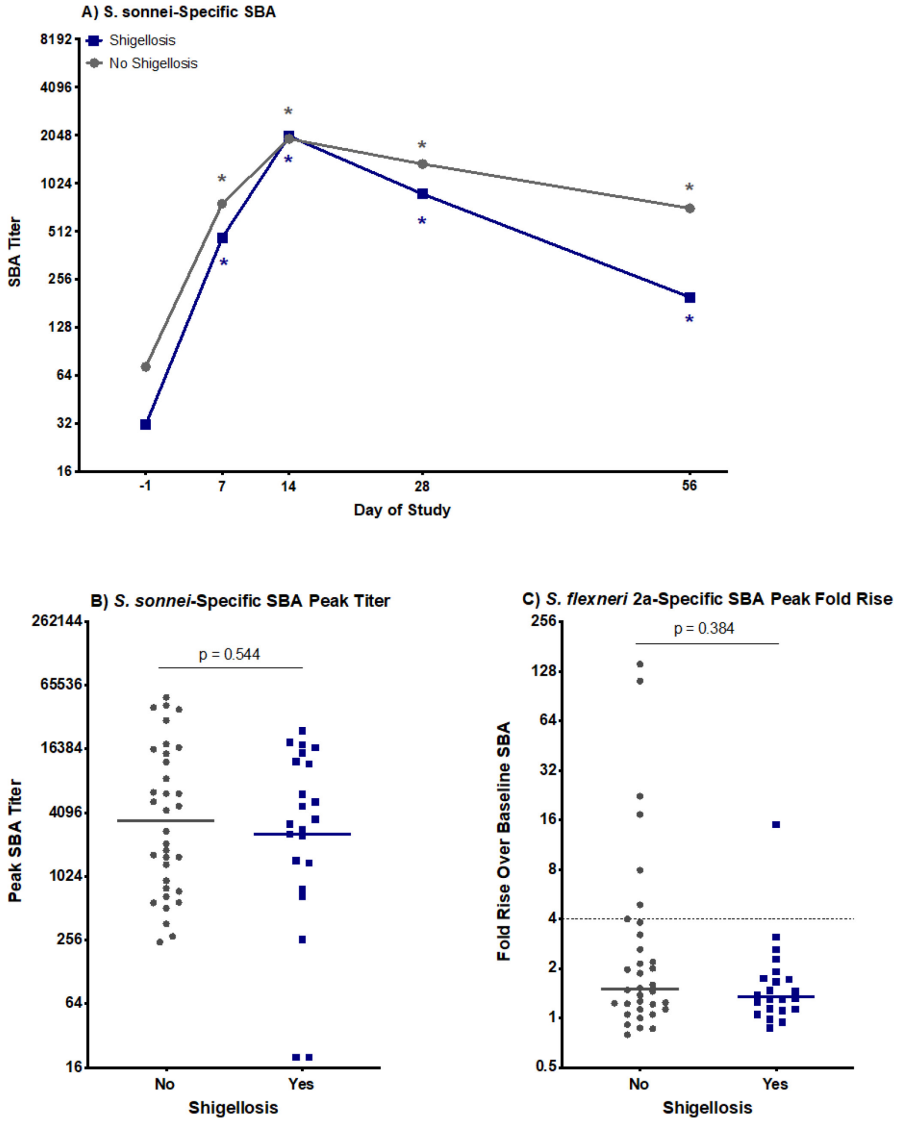
S. sonnei LPS-specific (A) fecal IgA and (B) IgG geometric mean ELISA endpoint titers at baseline/B and 3, 7 and 14 days post-challenge, grouped by subjects with (N=22) or without (N=34) shigellosis. * = significant difference as compared to baseline titers within shigellosis group; § = significant difference in titers between shigellosis groups at the same time point. Significance determined by 2-way ANOVA of log-transformed titers with Bonferroni post-hoc test. Spearman correlation of peak fold rise in (C) *S. sonnei* LPS-specific α4β7+ ALS and fecal IgA titers and (D) *S. sonnei* LPS-specific α4β7+ ALS and fecal IgG titers.

Figure 3.9. *S. sonnei* LPS and Invaplex-Specific Memory B Cell ALS IgG and IgA Responses



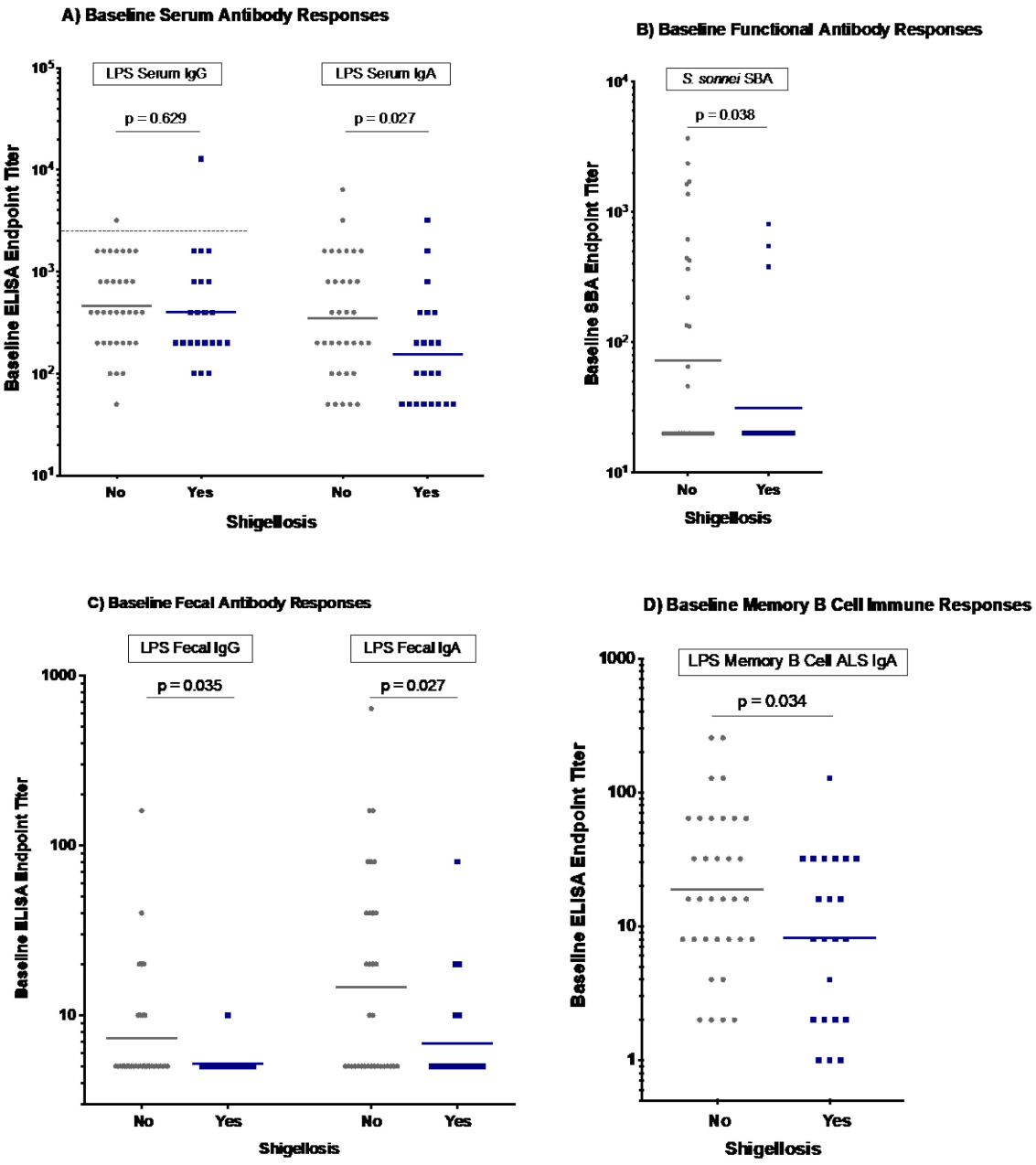
Tukey box and whisker plots of maximum fold-rise (day 28 or 56) over baseline memory B cell ALS ELISA endpoint titers, grouped by subjects with (N=22) or without (N=34) shigellosis. (A) *S. sonnei* LPS-specific memory B cell ALS IgA; (B) *S. sonnei* LPS-specific memory B cell ALS IgG; (C) *S. sonnei* IVP-specific memory B cell ALS IgA; (D) *S. sonnei* IVP-specific memory B cell ALS IgG. P-value determined by Mann-Whitney U test.

Figure 3.10. *S. sonnei* and *S. flexneri* 2a-Specific Serum Bactericidal Activity



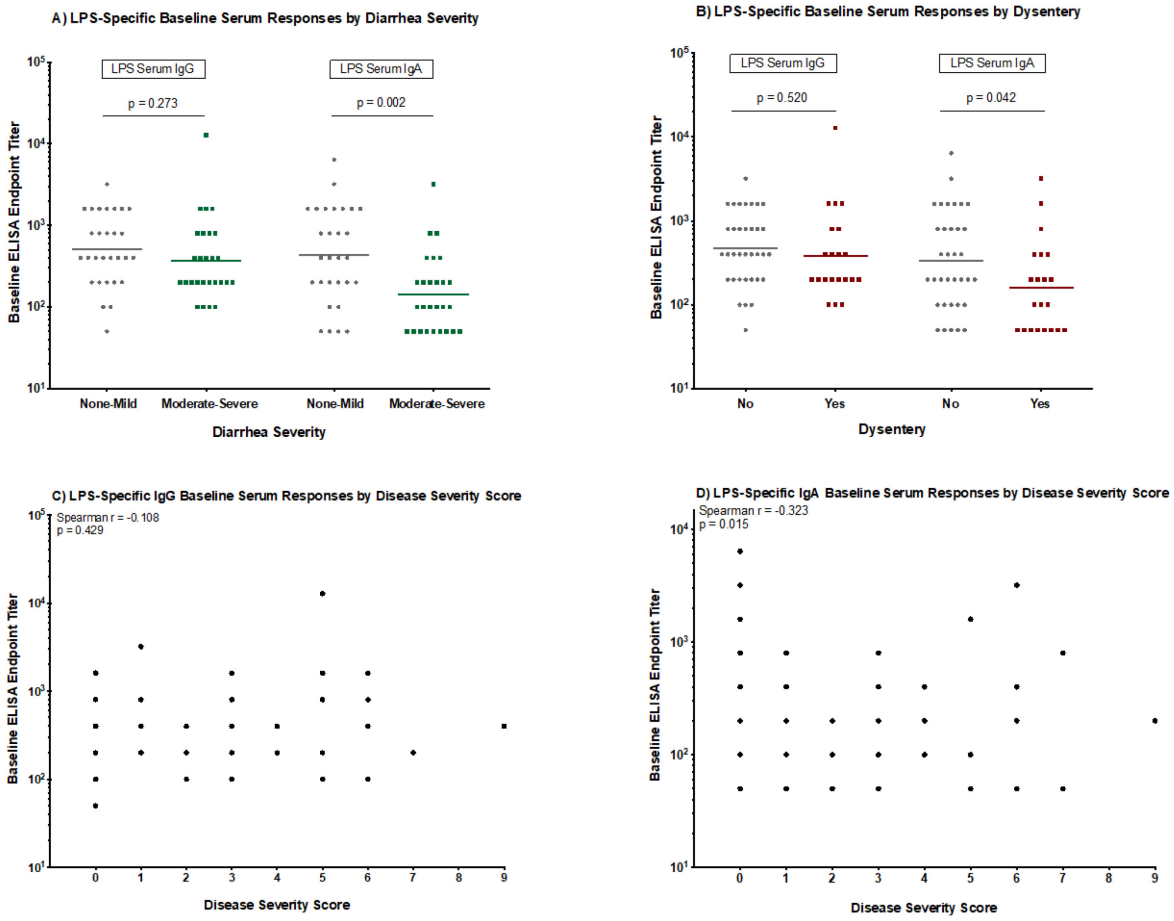
(A) Geometric mean *S. sonnei*-specific serum bactericidal titer (GMT) prior to challenge (day -1) and 7, 14, 28 and 56 days post-challenge, grouped by subjects with (N=22) or without (N=34) shigellosis. * = significant difference as compared to baseline titers within shigellosis group. Significance determined by 2-way ANOVA of log-transformed titers with Bonferroni post-hoc test. (B) Individual peak *S. sonnei*-specific SBA titers with group GMT grouped by subjects with (N=22) or without (N=34) shigellosis. P-value determined by T-test of log-transformed titers. (C) Individual fold-rise in *S. flexneri* 2a-specific SBA titers over baseline with group median grouped by subjects with (N=22) or without (N=34) shigellosis (line at 4 indicates cut-off for definition of a responder). P-value determined by Mann-Whitney U test.

Figure 3.11. *S. sonnei*-Specific Baseline Immune Responses Across Shigellosis Outcome



Individual ELISA or SBA endpoint titers with group geometric mean prior to challenge (baseline), grouped by subjects with (N=22) or without (N=34) shigellosis. (A) *S. sonnei* LPS-specific serum IgG (dashed line = 2500 cut-off titer used for study inclusion) and *S. sonnei* LPS-specific serum IgA, P-value determined by T-test of log-transformed titers; (B) *S. sonnei* serum bactericidal activity, P-value determined by Mann-Whitney U test; (C) *S. sonnei* LPS-specific fecal IgG and *S. sonnei* LPS-specific fecal IgA, P-value determined by Mann-Whitney U test; (D) *S. sonnei* LPS-specific memory B cell ALS IgA, P-value determined by T-test of log-transformed titers.

Figure 3.12. *S. sonnei* LPS-Specific Baseline Serum IgG and IgA Responses by Additional Disease Outcomes



Individual *S. sonnei* LPS-specific serum IgG or IgA ELISA endpoint titers with group geometric mean prior to challenge (baseline), grouped by (A) subjects with none-mild diarrhea (N=29) or moderate-severe diarrhea (N=27), and (B) subjects with (N=21) or without (N=35) dysentery. P-value determined by T-test of log-transformed titers. Spearman correlation of *S. sonnei* LPS-specific serum (C) IgG or (D) IgA baseline ELISA endpoint titers and disease severity score (N=56).

CHAPTER 4. IMMUNE RESPONSE CHARACTERIZATION IN A HUMAN CHALLENGE STUDY WITH A *SHIGELLA FLEXNERI* 2A BIOCONJUGATE VACCINE

The data presented in Chapter 4 has been submitted for publication to the journal Lancet Infectious Diseases and is undergoing peer-review:

Clarkson KA, Talaat K, Alaimo C, Martin P, Bourgeois AL, Dreyer A, Porter CK, Chakraborty S, Brubaker J, Elwood D, Frölich R, DeNearing B, Weerts HP, Feijoo B, Halpern J, Sack D, Riddle MS, Gambillara Fonck V, Kaminski RW. Immune Response Characterization in a Human Challenge Study with a *Shigella flexneri* 2a Bioconjugate Vaccine. Lancet Infect Dis 2020; Manuscript Submitted for Publication.

4.1. SUMMARY

Background. Diarrheal diseases are a leading cause of global morbidity and mortality affecting all ages, but especially children under the age of five in resource-limited settings. *Shigella* is a top tier bacterial pathogen contributing to diarrheal diseases and is considered a significant antimicrobial resistance threat. While improvements in hygiene, and access to clean water help as control measures, vaccination remains one of the most viable options for significantly reducing morbidity and mortality.

Methods. Flexyn2a is a bioconjugate vaccine manufactured using novel conjugation methodologies that enzymatically links the O-polysaccharide of *S. flexneri* 2a to exotoxin A of *Pseudomonas aeruginosa*. The protective capacity of Flexyn2a was assessed in a controlled human infection model after two intramuscular immunizations. Flexyn2a was well-tolerated and protected against severe illness post-oral challenge with *S. flexneri* 2a, 2457T. Immune responses post-immunization and post-oral challenge are described here.

Findings. Flexyn2a induced lipopolysaccharide (LPS)-specific serum IgG responses post-immunization that were associated with protection against shigellosis. Additionally, several other immune parameters, including memory B cell responses, bactericidal antibodies and serum IgA, were also elevated in vaccinees protected against shigellosis after oral challenge. Immunization with Flexyn2a also induced mucosal-homing, LPS-

specific IgG and IgA secreting B cells, indicating the vaccine induced immune effectors functioning at the site of intestinal infection.

Interpretation. Collectively, the results of these immunological investigations provide insights into protective immune mechanisms post-immunization with Flexyn2a which can be used to further guide vaccine development and may have applicability to the larger *Shigella* vaccine field.

Added Value of this Study. This is the first study to provide a thorough characterization of the systemic and mucosal immune responses induced after parenteral immunization with a *Shigella* conjugate vaccine, as well as to investigate the association of each immune parameter with protection from shigellosis. Additionally, this is the first study to report immune response data using the recently published consensus shigellosis endpoint, an outcome to be used in future *Shigella* challenge models and vaccine efficacy studies.

Implications of all the Available Evidence. The current study provides essential insights into protective immune mechanisms associated with parenteral immunization with a *Shigella* bioconjugate. The precedent set in the current study of thorough characterization of the immune responses post-immunization may influence the design and immunological analyses of future *Shigella* clinical studies and can help move the field towards the establishment of an immune correlate of protection for shigellosis.

4.2. INTRODUCTION

Shigella species are one of the leading causes of diarrhea-associated morbidity and mortality, accounting for millions of diarrhea-attributable deaths across all age groups annually.[1,23] Diarrheal disease morbidity and mortality estimates are highest among children under the age of 5, with *Shigella* being the second leading cause of diarrhea-associated mortality in this age group.[1,23] Furthermore, the impact of *Shigella* infection goes beyond the acute illness observed, as children with repeated enteric infections are also at risk of impaired cognitive performance and reduced height-for-age Z score.[16] Children impacted by this physical and cognitive stunting have also been estimated to be at a higher risk of mortality due to other infectious diseases.[17] Several additional post-infectious sequelae are also associated with *Shigella* infection, including post-infectious reactive arthritis and irritable bowel syndrome.[20,40]

Antibiotics are generally effective in the treatment of shigellosis; however, increasing rates of antibiotic-resistance[55,57] requires continual emphasis on primary prevention methods.[61] Along with improved access to clean water, increased sanitation and improved hygiene, vaccine development efforts are considered an integral piece of reducing the *Shigella* disease burden.

Epidemiological data and challenge/re-challenge studies have shown that prior *Shigella* infection protects from subsequent infection in a serotype-specific manner.[92,98] As *Shigella* serotypes are determined by the structure of their O-polysaccharide (OPS), lipopolysaccharide (LPS), or OPS alone, is considered to be a key protective antigen. Additionally, in order to protect against the most globally prevalent disease-causing

serotypes, an effective vaccine must be multi-valent. Although several *Shigella* vaccine approaches have been attempted in recent years, including live and killed whole cell vaccines, subcellular or subunit vaccines, as well as OPS conjugate vaccines,[157,158] there is currently no widely available licensed vaccine. When considering candidate vaccines, OPS conjugates are of particular interest as the strategy has a well-demonstrated safety and efficacy history.[123,125,188]

Flexyn2a is a bioconjugate vaccine manufactured using novel conjugation methodologies that enzymatically link the OPS of *S. flexneri* 2a to exotoxin A of *Pseudomonas aeruginosa* (EPA).[126,127] The *in vivo* conjugation process leads to a highly reproducible product that is simple, reliable and inexpensive to manufacture. Additionally, the *Shigella* bioconjugate vaccines have demonstrated robust safety and immunogenicity profiles in prior Phase 1 clinical assessments.[126,127] More recently, the Flexyn2a bioconjugate vaccine was assessed in a controlled human infection model (CHIM) to evaluate the preliminary efficacy following challenge with *S. flexneri* 2a strain 2457T, as described in Appendix B.

Promising efficacy results from the efficacy CHIM demonstrated the ability of Flexyn2a to protect against severe disease post-challenge with virulent *S. flexneri* 2a (Appendix B). Furthermore, robust *S. flexneri* 2a LPS-specific serum IgG and IgA responses were reported post-immunization, with serum IgG responses serving as a correlate of protection post-challenge (Appendix B). Efforts have since been underway to further characterize the immune responses induced post-parenteral immunization with the Flexyn2a bioconjugate, as well as post-oral challenge with *S. flexneri* 2a strain 2457T to

investigate potential correlates, or surrogates, of protection associated with *Shigella* infection. These analyses could further contribute to *Shigella* vaccine development efforts as well as enhance understanding of the mechanisms of protection associated with a parenterally administered *Shigella* bioconjugate vaccine. Moreover, data from the current challenge trial with the Flexyn2a bioconjugate were recently used to support the development of a consensus shigellosis endpoint and described herein is the first report of immune response analyses in a Phase 2b trial using this consensus definition.[166]

4.3. MATERIALS AND METHODS

Study Design and Efficacy. Trial design, conduct, randomization, masking and efficacy data are described in detail in Appendix B. Briefly, the trial was a randomized (1:1), double-blind, placebo-controlled study to assess preliminary efficacy post-immunization with Flexyn2a. Subject inclusion and exclusion criteria are detailed in Appendix B, along with subject screening and disposition. In an effort to enroll immunologically naïve subjects, subjects with serologic evidence of prior exposure to *S. flexneri* 2a, described as a *S. flexneri* 2a LPS-specific serum IgG titer of ≥2500, were excluded from participation, in addition to other exclusion criteria (Appendix B).

Subjects were intramuscularly administered either unadjuvanted Flexyn2a (n=34) or placebo (saline, n=33) on study days 0 and 28 (see Appendix B Figure 1). Each dose of Flexyn2a contained 10µg of *S. flexneri* 2a OPS and 50µg of EPA. One month after the last immunization (day 56), subjects (30 vaccinees, 29 placebo recipients) were orally challenged with approximately 1500 colony forming units of *S. flexneri* 2a strain 2457T. Five days post-challenge (or sooner if clinically warranted), subjects received antibiotics (ciprofloxacin or trimethoprim/sulfamethoxazole) twice daily for 3 days. Subjects were discharged from the inpatient facility after producing two consecutive culture negative stool samples over a period of two days, and after having received at least 2 doses of antibiotics. Demographic data and disease outcome data of enrolled subjects are described in detail in Appendix B.

Blood Processing. Whole blood for serum and peripheral blood mononuclear cells (PBMCs) was collected at multiple time points during the vaccination phase (study days 0, 7, 28, 35, and 56) as well as 3, 7 and 28 days post-challenge (study days 59, 63 and 84) (see Appendix B Figure 1). Serum samples were stored at -80±10˚C. PBMCs were isolated on a Ficoll gradient with Leucosep tubes (Grenier Bio-One), frozen and stored in liquid nitrogen until used in immunoassays.

Antibodies in Lymphocyte Secretions (ALS). Frozen PBMCs were thawed, washed and suspended in complete RPMI medium (10% heat inactivated fetal calf serum, 100 U/ml:100 μg/ml penicillin:streptomycin, 2 mM glutamine) at $5x10^6$ cells/ml, plated in a sterile 24-well tissue culture plate (1 ml/well) and cultured for 4 days at 37 ± 1°C with 5% CO_2 (cell viability not monitored). ALS supernatants were collected and frozen at -80 ± 10°C until used in immunoassays.

Enzyme-Linked Immunosorbent Assay (ELISA). Serum and ALS samples were assayed by ELISA to determine *S. flexneri* 2a LPS-specific antibody endpoint titers as previously described,[174] with the exception of the use of Immulon 1-B ELISA plates (Thermo Scientific) and human-specific secondary antibodies (reserve alkaline phosphatase (AP)-conjugated Goat-Anti-Human IgG, IgA or IgM; Seracare; AP-conjugated Mouse-Anti-Human IgG1, IgG2, IgG3 or IgG4; Southern Biotech). ELISA titers were defined as the reciprocal of the last dilution of serum with an optical density (OD) above the assay cut-off value. Samples that were negative at the starting dilution (the assay limit of detection (LOD)) were assigned a titer corresponding to half of the starting

dilution (½ LOD). Immune responders were defined a priori as having a ≥4-fold increase over their baseline titer.

α4β7 PBMC Separations. Frozen PBMCs from baseline (day 0), 7 days post-first immunization (day 7), as well as 3 and 7 days post-oral challenge (days 59 and 63) were thawed, washed and separated into α4β7 positive and negative PBMC populations as described in Chapter 3 using Miltenyi OctoMACS™ columns. The anti-α4β7 monoclonal antibody used for separation (Act-1; NIH AIDS Reagent Program) was conjugated to Alexa Fluor 647, allowing the purity of α4β7 populations to be assessed by flow cytometry. Post-separation, 100 µl from the α4β7+ and α4β7- populations for each subject/time point were analyzed using a FACSCanto II to ensure ≥90% purity in each of the α4β7+ or α4β7- populations (data not shown).

Memory B Cell Expansion and Quality Control. Frozen PBMCs from baseline (day 0), 28 days post-second immunization (day 56) and 28 days post-challenge (day 84) were thawed, washed and expanded as described in Chapter 3. After expansion, the cells were washed twice with mitogen-free complete RPMI medium, adjusted to $5x10^6$ cells/ml and cultured as described above to collect ALS from memory B cells. Successful expansion of memory B cell populations was assessed using flow cytometry as described above in Chapter 3.

Briefly, criteria for a successful expansion was defined as a post-expansion increase in cells positive for the CD19 B cell marker, as well as a ≥20% increase in B cells positive for the CD27 memory marker. Additionally, cell viability and cell concentrations were monitored pre- and post-expansion. Samples not meeting the criteria for successful

expansion of memory B cell populations were discarded and, using a new aliquot of frozen PBMCs, were expanded an additional time as outlined above. If the sample still did not meet the criteria for successful expansion, the sample was excluded from analyses. Additionally, memory B cell responses could only be determined for subjects with a sufficient amount of PBMCs available for the analysis: vaccinees (n=16/34), placebo recipients (n=27/33).

Serum Bactericidal Assay (SBA). Antibody functionality was assessed by determining *S. flexneri* 2a-specific bactericidal activity as outlined in Chapter 3 and as previously described.[175] Serum samples were titrated using serial 3-fold dilutions starting at 1:30 and titers were interpolated from a standard curve using NICE software.[176] A titer of 10, corresponding to one-third of the lowest serum dilution tested, was assigned to samples not exhibiting detectable bactericidal activity at the starting dilution. Immune responders were defined a priori as those with a ≥4-fold increase in bactericidal titer over baseline.

Disease Outcomes and Definitions. Immune responses pre- and post-challenge in subjects included in the challenge phase of the study (n=30 vaccinees and 29 placebo recipients) were compared across disease outcomes to evaluate the association of immune parameters with progression to, and severity of, disease. The majority of analyses were conducted using the recently developed consensus shigellosis CHIM endpoint.[166] In addition, as this consensus definition did not exist prior to study-protocol development, a small subset of immune parameters are also presented using the per

protocol a priori shigellosis definition (Appendix B) to compare immune responses across both definitions. All disease outcome definitions are described in detail in Table 3.1.

Statistical Analyses. Normally distributed continuous immune response data were analyzed using appropriate parametric tests (T-Test or repeated measures ANOVA) with Bonferroni post-hoc analyses as applicable. Non-normally distributed data were log-transformed prior to analysis in parametric tests, or appropriate non-parametric tests were used (Mann-Whitney U). ELISA titer cut-point analyses were performed by plotting receiver operating characteristics (ROC) and investigating sensitivity and specificity across different areas under the curve using Liu cut-point methods. All statistical tests were interpreted in a two-tailed fashion with p-values ≤ 0.05 considered statistically significant in Prism (Version 7 for MAC).

4.4. RESULTS

Serum IgG and IgA Responses. *S. flexneri* 2a LPS-specific serum IgG and IgA responses and percent seroconversions post-vaccination/challenge have been previously reported by treatment group (Appendix B, Table 5). Vaccinated subjects protected from shigellosis (regardless of the definition) had robust increases in serum IgG titers over baseline (all $p<0.0001$, 2-way ANOVA; Figure 4.1A and 4.2A); however, comparable significant increases over baseline were not observed in vaccinated subjects who developed shigellosis after challenge (all $p>0.999$, 2-way ANOVA; Figure 4.1A and 4.2A). In contrast, increases in LPS-specific serum IgA responses over baseline were observed in all vaccinated subjects, regardless of shigellosis outcome (all $p\leq0.05$, 2-way ANOVA; Figure 4.1B and 4.2B). Furthermore, while serum IgG responses on, and after, day 28 were higher in protected vaccinees compared to vaccinees with shigellosis, (all $p\leq0.01$, 2-way ANOVA; Figure 4.1A), only day 28 serum IgA titers were different across protected and unprotected vaccinees ($p=0.02$, 2-way ANOVA; Figure 4.1B). A second immunization with Flexyn2a did not increase the serum IgG or IgA responses for any of the vaccinated subjects, regardless of shigellosis outcome (Figure 4.1A-4.1B and 4.2A-B). In placebo recipients, no differences were observed over baseline in serum IgG or IgA titers across any of the shigellosis outcomes until after the day of challenge (day 56).

Oral challenge with *S. flexneri* 2a, 2457T did not significantly increase the LPS-specific serum IgG responses in vaccinated subjects, regardless of shigellosis outcome (all $p>0.999$, 2-way ANOVA; Figure 4.1A and 4.2A). Interestingly, while not statistically significant, serum IgA responses in unprotected vaccinees did increase by day 84 (all

p>0.999, 2-way ANOVA; Figure 4.1B and 4.2B), a trend not observed in protected vaccinees. Significant increases over baseline *S. flexneri* 2a LPS-specific serum IgG and IgA titers were observed post-challenge in placebo recipients progressing to shigellosis (all p≤0.05, 2-way ANOVA; Figure 4.1A and 4.1B). Similar magnitudes of increase in serum IgG or IgA responses were not observed in placebo recipients without shigellosis. Vaccinees protected from shigellosis had higher serum IgG responses on day 84 compared to all placebo recipients, regardless of shigellosis outcome (all p<0.0001, 2-way ANOVA; Figure 4.1A), whereas IgA responses in protected vaccinees were only higher compared to placebo recipients without shigellosis (p=0.008, 2-way ANOVA; Figure 4.1B).

Serum IgG and IgA titers on day of challenge predicted efficacy post-challenge. Using Liu cut-point analyses (Table 4.1), a serum IgG titer of 25,600 provided 66% efficacy while a lower serum IgA titer of 800 provided 54% efficacy in vaccinated subjects (Figure 4.1C and Table 4.1). Percent efficacy rose sharply thereafter with increasing serum IgG or IgA titers (Figure 4.1C and Table 4.2).

Serum IgG Subclass and IgM Responses. Immunization with Flexyn2a induced higher LPS-specific serum IgG1 responses compared to placebo recipients (all p≤0.01, 2-way ANOVA; Figure 4.3), an effect that appeared greatest among vaccinated subjects protected from shigellosis. Compared to vaccinees with shigellosis, protected vaccinees had higher serum IgG1 titers throughout the study (all p≤0.01, 2-way ANOVA; Figure 4.4B). Furthermore, serum IgG1 titers in unprotected vaccinees and all placebo recipients were similar in magnitude throughout the observation period (Figure 4.4B). Identical

trends were observed with serum IgG1 across the per protocol shigellosis definition (Figure 4.2C). Serum IgG1 titers on day of challenge were also associated with vaccine efficacy with a titer as low as 100 providing 70% efficacy in vaccinated subjects (Figure 4.4D and Table 4.1) which increases sharply to 85% when a titer of 200 is achieved (Figure 4.4D and Table 4.2).

S. flexneri 2a LPS-specific serum IgG2 responses showed a different trend compared to serum IgG1 responses with all vaccinated subjects having detectable increases in serum IgG2 responses, regardless of shigellosis outcome (Figure 4.2D and 4.4C). Although serum IgG2 titers were higher in protected vaccinees than in vaccinees with shigellosis, this difference was only significant on study day 28 ($p=0.039$, 2-way ANOVA; Figure 4.4C). Placebo recipients with shigellosis had increased LPS-specific serum IgG2 responses post-challenge ($p=0.036$, 2-way ANOVA; Figure 4.4C); however, a similar magnitude of response in placebo recipients without shigellosis was not observed (Figure 4.4C). When comparing serum IgG2 responses across per protocol shigellosis, similar trends are observed with the exception of the difference between protected and unprotected vaccinees remaining significant different beyond day 28 (Figure 4.2D). Serum IgG2 titers at time of challenge (day 56) were associated with percent efficacy in vaccinated subjects however, at a higher magnitude of response as compared to serum IgG1. A serum IgG2 titer of 800 provided 56% efficacy (Figure 4.4D and Table 4.1) while a 4-fold increase in serum IgG2 titer to 3,200 provided 76% efficacy (Figure 4.4D and Table 4.2).

Immunization with Flexyn2a induced low but significant *S. flexneri* 2a LPS-specific serum IgM titers 28 days post-second immunization (p=0.23, 2-way ANOVA; Figure 4.3A). When vaccinated subjects are further grouped by shigellosis outcome, the significant increase in serum IgM on day 56 only remains for protected vaccinees (p=0.041, 2-way ANOVA; Figure 4.4A). A similar magnitude of increase was observed in the serum IgM responses of placebo recipients 28 days post-challenge (p=0.016, 2-way ANOVA; Figure 4.3A). As IgM is an efficient complement activator, it is important to consider that serum IgM levels induced post-vaccination may play an important role in bactericidal activity and influence the observed responses. Minimal to undetectable increases in LPS-specific serum IgG3 and IgG4 responses were observed throughout the study with no significant differences across treatment groups at any time point (Figure 4.3D and 4.3E).

Bactericidal Responses. Flexyn2a induced robust *Shigella*-specific bactericidal activity after one immunization, with responses in the vaccinated group remaining elevated over baseline through study day 84 (all p<0.0001, 2-way ANOVA; Figure 4.5A). While vaccinees protected from shigellosis had higher SBA titers compared to unprotected vaccinees, this difference was not statistically significant (Figure 4.5B). Across all vaccinated subjects, neither a second immunization with Flexyn2a, nor oral challenge with *S. flexneri* 2a, 2457T increased the overall magnitude of the bactericidal responses (Figure 4.5A and 4.5B); however, a second immunization with Flexyn2a did increase the frequency of responders from 70% to 83% (data not shown). Placebo recipients progressing to shigellosis showed a similar magnitude of increase in bactericidal responses by day 84 with responses in these individuals surpassing those

observed in unprotected vaccinees but not those observed in protected vaccinees (Figure 4.5B). The relationship between percent efficacy and bactericidal titer increased steadily with an SBA titer of 3,415 providing 64% efficacy (Figure 4.5C and Table 4.1). When the bactericidal titer is increased to 13,933, the percent efficacy also increases 79% (Figure 4.5C and Table 4.2).

α4β7 ALS Responses. Flexyn2a induced significant increases in both α4β7+ and α4β7- ALS responses 7 days post-first immunization (all p≤0.01, 2-way ANOVA; Figure 4.6). Protected vaccinees had higher α4β7+ ALS IgG responses on day 7 compared to unprotected vaccinees (p=0.0002, 2-way ANOVA; Figure 4.7A). Additionally, α4β7+ ALS IgG responses in protected vaccinees were higher 7 days post-first immunization compared to 7 days post-oral challenge with *S. flexneri* 2a, 2457T (p=0.004, 2-way ANOVA; Figure 4.7A). In unprotected vaccinees a minimal, non-significant boost in α4β7+ ALS IgG responses from day 7 to day 63 was observed (Figure 4.7A). Flexyn2a also increased the α4β7+ ALS IgA responses post-first vaccination; however, there were no differences between protected and unprotected vaccinated subjects (Figure 4.7B). The α4β7+ ALS IgA responses post-challenge showed similar trends to the α4β7+ ALS IgG responses, with protected vaccinees having higher α4β7+ ALS IgA responses on day 7 compared to day 63 (p=0.044, 2-way ANOVA; Figure 4.7B) while oral challenge increased the α4β7+ ALS IgA responses in unprotected vaccinees (Figure 4.7B).

Vaccinated subjects protected from shigellosis had robust α4β7- ALS IgG responses 7 days post-first immunization as compared to unprotected vaccinees (p=0.0002, 2-way ANOVA; Figure 4.8A); however, a similar magnitude of increase in

α4β7- ALS IgA responses was not observed (Figure 4.8B). Both α4β7+ and α4β7- antibody titers were associated with percent efficacy with any increase (≥2-fold rise over baseline) in α4β7+ or α4β7- antibody titer associated with 60-70% efficacy (Figures 4.7C and 4.8C and Tables 4.1 and 4.2). LPS-specific α4β7+ IgG responses quickly reached 86% efficacy at a low titer of 4 while the α4β7- IgG and α4β7 IgA responses did not reach this same level of efficacy until a titer of between 8-32 was reached (Figures 4.7C and 4.8C and Tables 4.1 and 4.2).

Memory B Cell Responses. Immunization with Flexyn2a induced robust *S. flexneri* 2a LPS-specific memory B cell IgG responses by day 56 (p=0.02, 2-way ANOVA; Figure 4.9A) which further increased post-challenge. Memory B cell IgG responses in vaccinated subjects on day of challenge (day 56) were comparable to the memory B cell IgG responses detected in placebo recipients post-challenge (day 84) (Figure 4.9A). Flexyn2a also increased the LPS-specific memory B cell IgA responses post-immunization; however, increases over baseline were not significant until after subjects were challenged with *S. flexneri* 2a, 2457T (p=0.004, 2-way ANOVA; Figure 4.9B).

Correlation of Immune Parameters in Vaccinated Subjects. Not surprisingly, the strongest correlation in immune parameters among vaccinated subjects was between α4β7+ ALS IgG and IgA responses (Spearman r=0.87; Figure 4.10) while α4β7- ALS IgG and IgA were correlated at a lower level (Spearman r=0.51; Figure 4.10). With the exception of the α4β7- ALS IgG responses, which were most strongly correlated with serum IgG1 (Spearman r=0.61; Figure 4.10), all other α4β7+ and α4β7- ALS responses showed a moderate correlation with serum IgA (Spearman r=0.64-0.73; Figure 4.10).

Serum IgA also correlated with serum IgG1 (Spearman r=0.65; Figure 4.10) and IgG2 (Spearman r=0.63; Figure 4.10); however, the immune parameters with the strongest serum IgA correlation was α4β7+ ALS IgG (Spearman r=0.73; Figure 4.10) and serum IgG (Spearman r=0.72; Figure 4.10).

Of the serum IgG subclass responses, IgG2 was most strongly correlated with serum total IgG (Spearman r=0.82; Figure 4.10) whereas serum IgG1 was most highly correlated serum IgA followed by α4β7+ ALS IgG and α4β7- ALS IgG responses (both Spearman r=0.61; Figure 4.10). Bactericidal activity was most strongly correlated with α4β7+ ALS IgG responses (Spearman r=0.58; Figure 4.10) followed by serum IgG1 responses (Spearman r=0.46; Figure 4.10). Memory B cell IgG and IgA responses correlated best with each other (Spearman r=0.66; Figure 4.10) however, memory B cell IgG also correlated with serum IgG and IgA responses (Spearman r=0.53-0.54; Figure 4.10). Aside from the correlation with memory IgG responses, memory B cell IgA responses correlated best with serum IgA responses (Spearman r=0.62; Figure 4.10), as well as α4β7- ALS IgA responses (Spearman r=0.59; Figure 4.10).

4.5. DISCUSSION

Immunization with one dose of Flexyn2a elicited robust *S. flexneri* 2a LPS-specific serum IgG and IgA antibody responses, as well as functional bactericidal antibody responses. Interestingly, while Flexyn2a induced both LPS-specific serum IgG1 and IgG2 responses, serum IgG1 responses were more closely correlated with protection from shigellosis as compared to serum IgG2, regardless of the higher magnitude of serum IgG2 titers observed post-vaccination. There was an increase in serum IgG2 levels post-challenge in placebo recipients but no increases in serum IgG1. When considering overall magnitude of the response, the IgG subclass titers observed in this study confirm previously documented differences across IgG1 and IgG2 responses based on *Shigella* serotype, with *S. flexneri* 2a and *S. sonnei* inducing either an IgG2 or IgG1 dominated response, respectively.[124,189,190] However, in the current study, when the focus is placed on the association of each IgG subclass with protection from shigellosis rather than overall magnitude of response, a potentially important role for *Shigella*-specific serum IgG1 in protection from shigellosis post-parenteral immunization is suggested. As IgG1 antibodies are highly efficient at activating complement as compared to IgG2, [191] they may be contributing to the killing of shigellae at a higher level compared to IgG2 while in the intestinal lamina propria during the process of transudation. The increased complement activation efficiency of IgG1 antibodies may therefore require a lower magnitude or threshold of response for them to be effective. Additionally, the role of IgG1 antibodies may be especially important in the context of the α4β7+ ALS IgG

responses observed in this study, which is further evidenced by the correlation between these two parameters.

Indeed, another important finding in this study is the induction post-vaccination of *Shigella* LPS-specific α4β7+ ALS IgG and IgA secreting B cells which are likely homing to the gut. A traditionally accepted paradigm in regard to parenterally delivered vaccines in the context of mucosal pathogens is their inability to induce mucosal immune responses, making these vaccines reliant on robust systemic responses leading to transudation of systemic antibodies into mucosal effector sites.[180,192,193] While this paradigm has been previously challenged in the context of other pathogen-specific parenterally delivered vaccines such as influenza, polio and tetanus,[194-196] mucosal immune responses observed in these, and similar, studies may be a result of a secondary antigen exposure after an initial oral priming exposure.[192,197] Furthermore, investigations of mucosal immune responses have largely focused on the importance of IgA secreting gut-homing B cells or secretory IgA in mucosal secretions.[193,198,199] While there is no doubt that secretory IgA plays a vital role in protection against mucosal pathogens,[200] investigations into the protective capacity of antigen-specific IgG secreting α4β7+ B cells against mucosal pathogens have been limited.

Parenteral immunization with the Flexyn2a bioconjugate vaccine induced robust LPS-specific α4β7+ ALS IgG responses in vaccinated subjects protected from shigellosis. While lower in magnitude, vaccinees also had increased α4β7+ ALS IgA titers, demonstrating the ability of Flexyn2a to induce both an IgG and IgA α4β7+ immune response. To our knowledge, this is the first study to report antigen-specific B cells

positive for the gut-homing marker post-parenteral immunization with conjugate vaccine for an enteric pathogen. The induction of α4β7+ IgG and IgA secreting B cells post-immunization may offer a mechanistic explanation of the protection afforded by the bioconjugate vaccine as LPS-specific B cells may home to the gut and produce antibodies at the site of infection, which can be actively secreted (IgA) or passively transudated (IgG) into the lumen. This finding could be especially important in the context of the α4β7+ LPS-specific IgG secreting B cells as these antibodies may contribute to the neutralization, or direct killing (either via complement activation or opsonization) of shigellae in the lamina propria that have transcytosed across the intestinal epithelial barrier. Additionally, the immune responses induced after natural infection or pre-existing immunity may offer different mechanisms of protection.

Similar investigations into the LPS-specific α4β7+ IgG and IgA responses were conducted on samples from the Phase 1 clinical study of Flexyn2a administered with and without Alum.[127] Results from the Phase 1 α4β7 analyses (data not reported) mirrored the responses achieved in the current study, further confirming the ability of Flexyn2a to induce an antigen-specific α4β7+ antibody response. Although the addition of Alum did not influence the magnitude of the LPS-specific α4β7+ ALS IgG and IgA response or the number of responders, it is important to consider the possibility that alternative adjuvants with enhanced capacity to augment mucosal immune responses or influence the phenotype of the immune response may provide different results, warranting further investigations.[201] Therefore, as recently recommended,[164] future studies should investigate the IgG subclass responses as well as the bactericidal activity in α4β7+ ALS

samples. Determining the dominant subclass of the α4β7+ IgG secreting B cells and the bactericidal ability of these antibodies could further inform mechanisms of protection during *Shigella* infection.

A second immunization with Flexyn2a or oral challenge with *S. flexneri* 2a, 2457T did not substantially boost any of the tested immune responses in protected vaccinees. In contrast, challenge with *S. flexneri* 2a, 2457T increased multiple *Shigella*-specific immune responses in unprotected vaccinees as well as naïve subjects, specifically in serum IgA, serum IgG2 and α4β7+ IgA responses. The post-challenge immune response dampening in protected vaccinees may be due to a reduced duration of antigenic exposure during the challenge phase suggesting that, in some cases, Flexyn2a is capable of reducing the number of shigellae that are able to invade the mucosal epithelium. Additionally, antibodies in the lamina propria may also work to reduce the number of shigellae that are able to invade the basolateral surface of intestinal epithelial cells. Interestingly, this phenomenon of an immune response dampening has also been observed in other enteric human challenge studies.[202,203]

As subjects were screened with multiple *Shigella*-specific exclusion criteria, including serological evidence of prior exposure to *S. flexneri* 2a, it was expected that the placebo subjects would progress to disease post-challenge. Nonetheless, only 59% and 62% of the placebo recipients met the consensus and per protocol shigellosis endpoint, respectively, similar to previous reports.[169,183,189] Prior *S. sonnei* and ETEC CHIMs have identified increased serum IgA titers to key antigens at baseline as predictive of post-challenge disease risk.[183,189] Similar investigations were performed in the current study

however no differences were observed in the pre-challenge serum IgA responses of placebo subjects across shigellosis outcome. Interestingly, when additional baseline immune parameters were investigated, naïve subjects not progressing to shigellosis were found to have significantly higher LPS-specific memory B cell IgA responses on the day of challenge as compared to placebo subjects with shigellosis (p=0.035, T-Test of log-transformed titers; data not shown). This difference in LPS-specific memory B cell IgA response prior to challenge has also been recently reported in a *S. sonnei* CHIM.[189] While no other differences in immune responses on day of challenge were observed, these data, as well as in other CHIMs, demonstrate the importance of additional investigations into reduced disease risk post-challenge as this may have a substantial effect in the assessment of vaccine efficacy.

Consistent with results from the Phase 1 study of Flexyn2a,[127] and other clinical assessments of *Shigella* conjugate vaccines,[123,188] parenteral immunization with the bioconjugate induced robust systemic immune responses. Flexyn2a also induced functional antibody responses as well as LPS-specific memory and α4β7+ B cells. This study also confirmed the vaccine's previously reported excellent safety profile and demonstrated the vaccine's ability to induce a protective immune response in a *S. flexneri* 2a CHIM setting (Appendix B).[126,127,204] The combination of a robust safety and immunogenicity profile, with efficacy against oral challenge and a simple, affordable and reproducible manufacturing process[205,206] supported the cGMP production of a quadrivalent *Shigella* bioconjugate vaccine formulation. The quadrivalent formulation is currently undergoing safety and immunogenicity evaluations in an age-descending trial in

Kenya (ClinicalTrials.gov Identifier: NCT04056117). Promising results achieved in the age-descending trial may support pivotal field trials of the final vaccine construct.

Table 4.1. Liu Analysis Cut-Points across Different LPS-Specific Immune Parameters Differentiating Vaccinated Subjects with Shigellosis from Vaccinated Subjects without Shigellosis Post-Challenge

Immune Parameter	ROC AUC[a]	Optimal Cut-Point[b]	Shigellosis Outcome (n)			Shigellosis Risk Estimate		Vaccinees Meeting Cut-Point	
			With	Without	Total	Relative Risk[c]	P-Value[d]	% Efficacy[e]	P-Value[f]
Serum IgG									
Day 28 Titer	0.805	≥12800	5	16	21	**0.357**	**0.042**	**59.4%**	**0.021**
		<12800	6	3	9				
Day 56 Titer	0.792	≥25600	3	12	15	0.375	0.128	**65.9%**	**0.025**
		<25600	8	7	15				
Max Titer	0.755	≥12800	7	17	24	0.438	0.156	50.2%	0.052
		<12800	4	2	6				
Peak Fold Rise	0.842	≥8	4	16	20	**0.286**	**0.015**	**65.9%**	**0.009**
		<8	7	3	10				
Serum IgA									
Day 28 Titer	0.718	≥800	5	16	21	**0.357**	**0.042**	**59.4%**	**0.021**
		<800	6	3	9				
Day 56 Titer	0.726	≥800	6	16	22	0.436	0.104	**53.5%**	**0.046**
		<800	5	3	8				
Max Titer	0.752	≥1600	4	15	19	**0.331**	**0.047**	**64.1%**	**0.017**
		<1600	7	4	11				
Peak Fold Rise	0.674	≥8	6	17	23	0.365	0.068	**55.5%**	**0.026**
		<8	5	2	7				
Serum IgG1									
Day 28 Titer	0.742	≥100	1	11	12	**0.150**	**0.018**	**85.8%**	**0.005**
		<100	10	8	18				
Day 56 Titer	0.761	≥100	3	14	17	**0.287**	**0.023**	**69.9%**	**0.013**
		<100	8	5	13				
Max Titer	0.804	≥100	3	15	18	**0.250**	**0.009**	**71.6%**	**0.006**
		<100	8	4	12				
Peak Fold Rise	0.796	≥2	3	15	18	**0.250**	**0.009**	**71.6%**	**0.006**
		<2	8	4	12				
Serum IgG2									
Day 28 Titer	0.691	≥400	8	17	25	0.533	0.327	45.4%	0.061
		<400	3	2	5				
Day 56 Titer	0.699	≥800	6	17	23	0.365	0.068	**55.5%**	**0.026**
		<800	5	2	7				
Max Titer	0.711	≥800	6	17	23	0.365	0.068	**55.5%**	**0.026**
		<800	5	2	7				
Peak Fold Rise	0.803	≥4	5	16	21	**0.357**	**0.042**	**59.4%**	**0.021**
		<4	6	3	9				
Serum Bactericidal Activity									
Day 28 Titer	0.641	≥3162	5	13	18	0.556	0.266	52.6%	0.070
		<3162	6	6	12				
Day 56 Titer	0.780	≥3415	4	15	19	**0.331**	**0.047**	**64.1%**	**0.017**
		<3415	7	4	11				
Max Titer	0.708	≥2561	6	17	23	0.365	0.068	**55.5%**	**0.023**
		<2561	5	2	7				
Peak Fold Rise	0.763	≥6	5	15	20	0.417	0.108	**57.4%**	**0.040**
		<6	6	4	10				
α4β7+ and α4β7- ALS IgG and IgA (Day 7 Titer)									
α4β7+ IgG	0.882	≥2	3	17	20	**0.150**	**0.0004**	**74.4%**	**0.003**
		<2	6	0	6				
α4β7- IgG	0.797	≥2	3	15	18	**0.222**	**0.008**	**71.6%**	**0.006**
		<2	6	2	8				
α4β7+ IgA	0.696	≥2	3	12	15	0.367	0.103	**65.9%**	**0.025**
		<2	6	5	11				
α4β7- IgA	0.647	≥2	2	8	10	0.457	0.399	**65.9%**	**0.065**
		<2	7	9	16				
Memory B Cell ALS IgG and IgA (Day 56)									
IgG	0.729	≥32	4	10	14	0.653	0.466	51.3%	0.104
		<32	7	9	16				
IgA	0.590	≥64	7	15	22	0.636	0.417	45.7%	0.089
		<64	4	4	8				

[a] Area under the curve (AUC) of a receiver operator characteristic (ROC) graph

[b] Liu cut-point analysis used to determine the optimal point in the AUC which maximizes the product of sensitivity and specificity of the ROC.

[c] Calculated as: (shigellosis rate among vaccinees at or above optimal cut-point / shigellosis rate among vaccinees below optimal cut-point).

[d] P-value determined by Fisher's Exact Test comparing shigellosis rate among vaccinees at or above optimal cut-point versus shigellosis rate among vaccinees below optimal cut-point.

[e] Calculated as: [1 – (shigellosis rate among vaccinees at or above optimal cut-point / shigellosis rate among placebo recipients)] x 100.

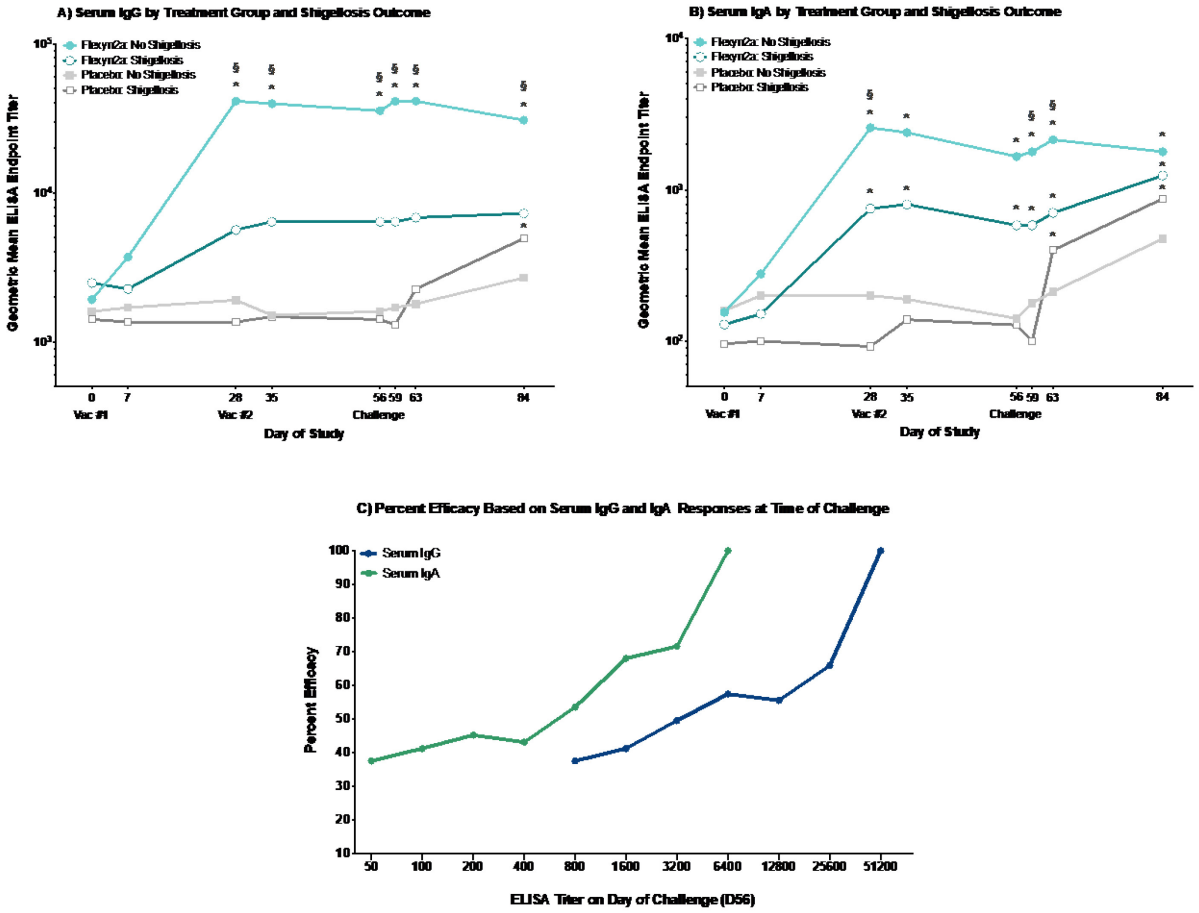
[f] P-value determined by Fisher's Exact Test comparing shigellosis rate among vaccinees at or above optimal cut-point versus shigellosis rate among placebo recipients.

Table 4.2. Cut-Points Providing ~70-80% Efficacy across Different LPS-Specific Immune Parameters Differentiating Vaccinated Subjects with Shigellosis from Vaccinated Subjects without Shigellosis Post-Challenge

Immune Parameter	ROC AUC[a]	Titer Cut-Point[b]	Shigellosis Outcome (n)			Shigellosis Risk Estimate		Vaccinees Meeting Cut-Point	
			With	Without	Total	Relative Risk[c]	P-Value[d]	% Efficacy[e]	P-Value[f]
Serum IgG									
Day 28 Titer	0.805	≥25600	2	12	14	**0.254**	**0.026**	**75.6%**	**0.009**
		<25600	9	7	16				
Day 56 Titer	0.792	≥25600	3	12	15	0.375	0.128	**65.9%**	**0.025**
		<25600	8	7	15				
Max Titer	0.755	≥51200	1	9	10	**0.200**	**0.049**	**82.9%**	**0.011**
		<51200	10	10	20				
Peak Fold Rise	0.842	≥16	3	14	17	**0.287**	**0.023**	**69.9%**	**0.013**
		<16	8	5	13				
Serum IgA									
Day 28 Titer	0.718	≥3200	3	13	16	0.328	0.057	**68.0%**	**0.013**
		<3200	8	6	14				
Day 56 Titer	0.726	≥3200	2	10	12	0.333	0.121	**71.6%**	**0.019**
		<3200	9	9	18				
Max Titer	0.752	≥3200	3	15	18	**0.250**	**0.009**	**71.6%**	**0.006**
		<3200	8	4	12				
Peak Fold Rise	0.674	≥16	4	15	19	**0.331**	**0.047**	**64.1%**	**0.017**
		<16	7	4	11				
Serum IgG1									
Day 28 Titer	0.742	≥100	1	11	12	**0.150**	**0.018**	**85.8%**	**0.005**
		<100	10	8	18				
Day 56 Titer	0.761	≥200	1	10	11	**0.173**	**0.023**	**84.5%**	**0.011**
		<200	10	9	19				
Max Titer	0.804	≥200	1	12	13	**0.131**	**0.007**	**86.9%**	**0.002**
		<200	10	7	17				
Peak Fold Rise	0.796	≥4	1	11	12	**0.150**	**0.018**	**85.8%**	**0.005**
		<4	10	8	18				
Serum IgG2									
Day 28 Titer	0.691	≥1600	3	9	12	0.563	0.442	57.4%	0.085
		<1600	8	10	18				
Day 56 Titer	0.699	≥3200	1	6	7	0.329	0.215	75.6%	0.088
		<3200	10	13	23				
Max Titer	0.711	≥3200	1	6	7	0.329	0.215	75.6%	0.088
		<3200	10	13	23				
Peak Fold Rise	0.803	≥8	2	14	16	**0.194**	**0.007**	**78.7%**	**0.004**
		<8	9	5	14				
Serum Bactericidal Activity									
Day 28 Titer	0.641	≥13806	4	11	15	0.571	0.450	54.5%	0.060
		<13806	7	8	15				
Day 56 Titer	0.780	≥13933	1	7	8	0.275	0.199	**78.7%**	**0.042**
		<13933	10	12	22				
Max Titer	0.708	≥15814	1	7	8	0.275	0.199	**78.7%**	**0.021**
		<15814	10	12	22				
Peak Fold Rise	0.763	≥15	2	9	11	0.384	0.140	**69.0%**	**0.034**
		<15	9	10	19				
α4β7+ and α4β7- ALS IgG and IgA (Day 7 Titer)									
α4β7+ IgG	0.882	≥4	1	11	12	**0.146**	**0.014**	**85.8%**	**0.005**
		<4	8	6	14				
α4β7- IgG	0.797	≥2	3	15	18	**0.222**	**0.008**	**71.6%**	**0.006**
		<2	6	2	8				
α4β7+ IgA	0.696	≥8	1	7	8	0.281	0.190	**78.7%**	**0.042**
		<8	8	10	18				
α4β7- IgA	0.647	≥4	1	6	7	0.339	0.357	75.6%	0.088
		<4	8	11	19				
Memory B Cell ALS IgG and IgA (Day 56)									
IgG	0.729	≥64	1	8	9	0.233	0.100	**81.0%**	**0.021**
		<64	10	11	21				
IgA	0.590	≥64	7	15	22	0.636	0.417	45.7%	0.089
		<64	4	4	8				

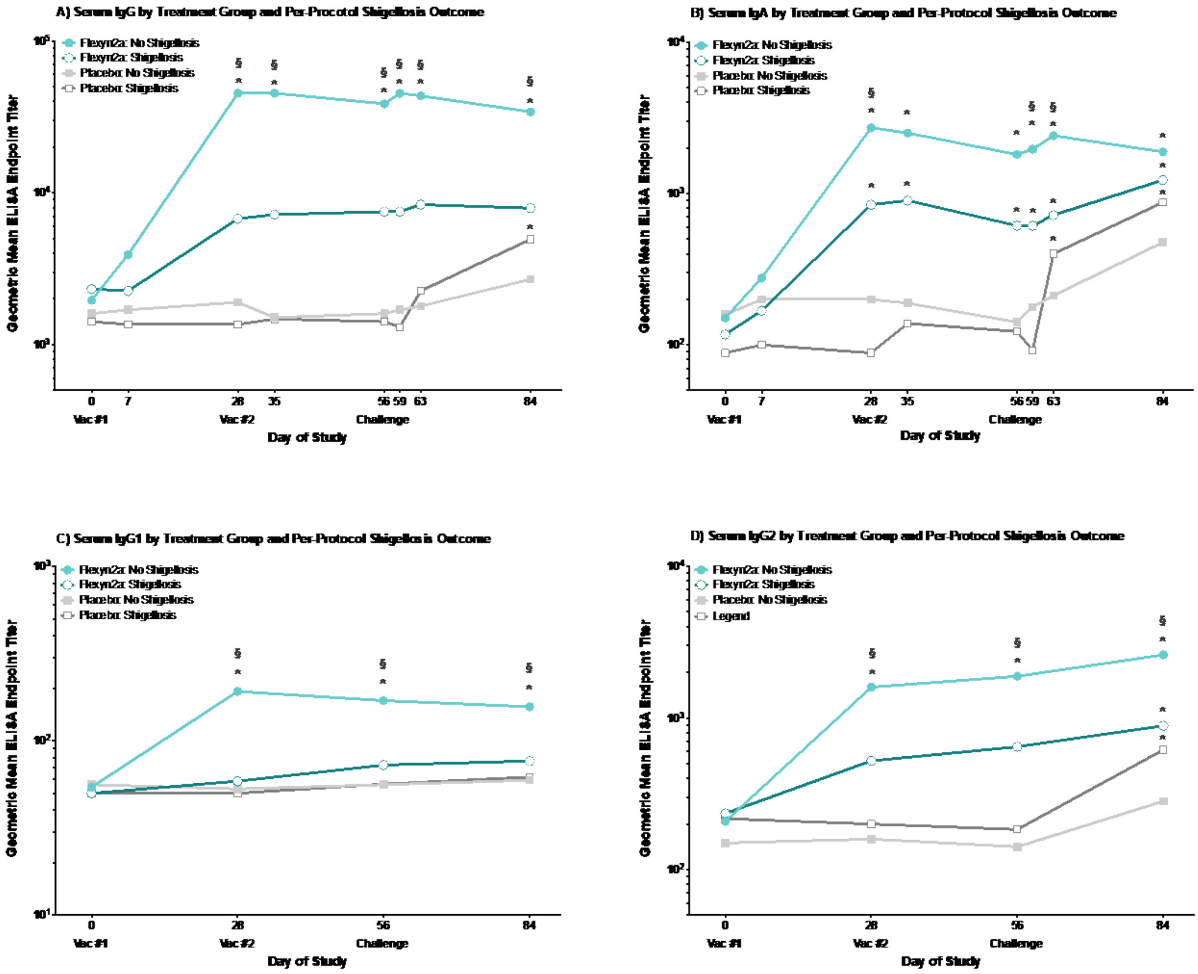
[a] Area under the curve (AUC) of a receiver operator characteristic (ROC) graph
[b] Optimal cut-point chosen based on the titer that provided as close to or between 70-80% efficacy.
[c] Calculated as: (shigellosis rate among vaccinees at or above optimal cut-point / shigellosis rate among vaccinees below optimal cut-point).
[d] P-value determined by Fisher's Exact Test comparing shigellosis rate among vaccinees at or above optimal cut-point versus shigellosis rate among vaccinees below optimal cut-point.
[e] Calculated as: [1 – (shigellosis rate among vaccinees at or above optimal cut-point / shigellosis rate among placebo recipients)] x 100.
[f] P-value determined by Fisher's Exact Test comparing shigellosis rate among vaccinees at or above cut-point versus shigellosis rate among placebo recipients.

Figure 4.1. *S. flexneri* 2a LPS-Specific Serum IgG and IgA Responses by Shigellosis Outcome



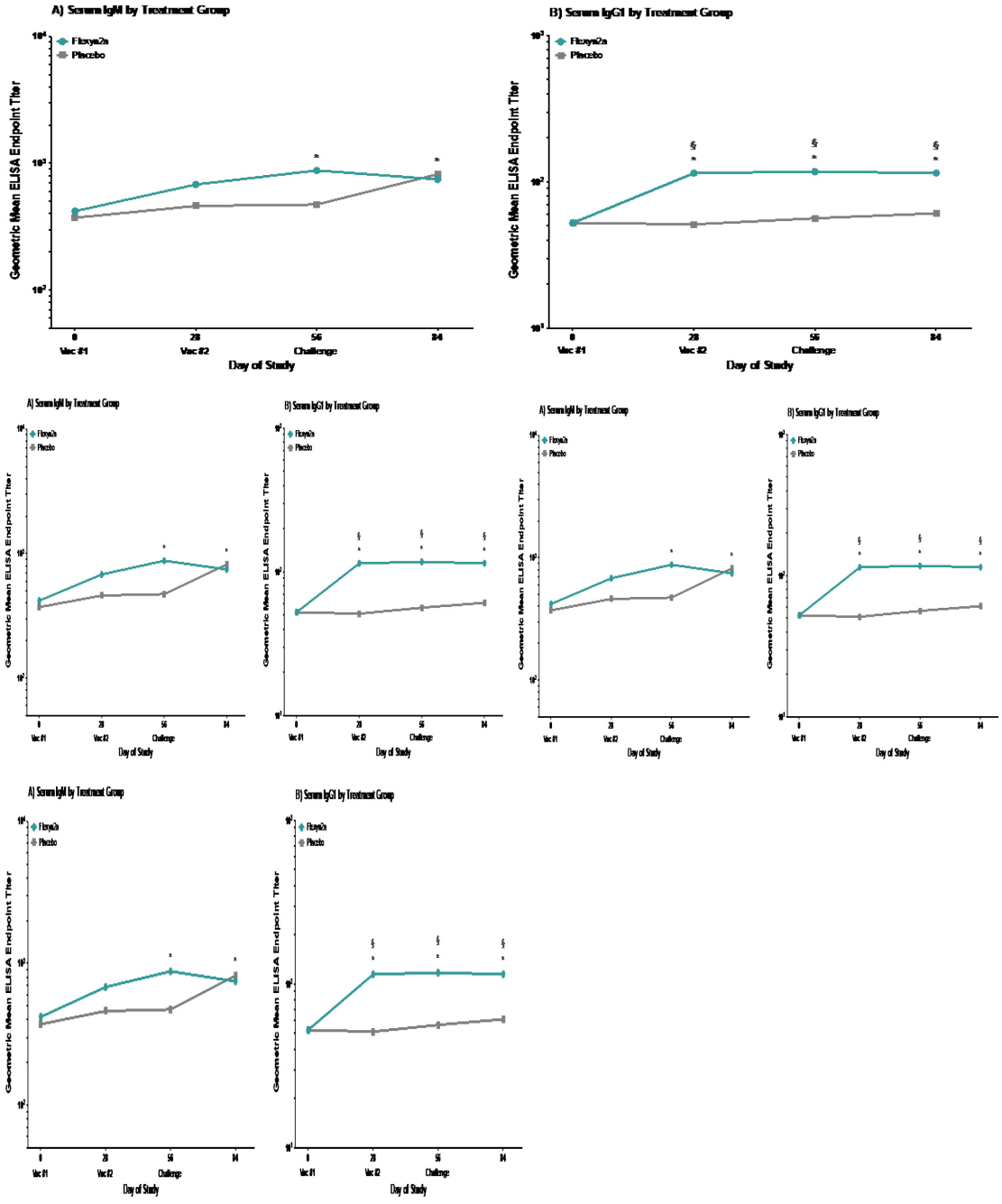
(A) *S. flexneri* 2a LPS-specific serum IgG responses grouped by vaccinated subjects with (N=11) or without (N=19) consensus shigellosis, and placebo subjects with (N=17) or without (N=12) consensus shigellosis. (B) *S. flexneri* 2a LPS-specific serum IgA responses grouped by vaccinated subjects with (N=11) or without (N=19) consensus shigellosis, and placebo subjects with (N=17) or without (N=12) consensus shigellosis. * = significant difference as compared to baseline titers within the same treatment group/shigellosis outcome. § = significant difference in titers within treatment group across shigellosis outcome at the same time point. Significance determined by 2-way ANOVA of log-transformed titers with Bonferroni post-hoc test. (D) Percent efficacy post-challenge in vaccinated subjects across increasing serum IgG and IgA ELISA endpoint titers.

Figure 4.2. *S. flexneri* 2a LPS-Specific Serum Antibody Responses by Per-Protocol Shigellosis Outcome



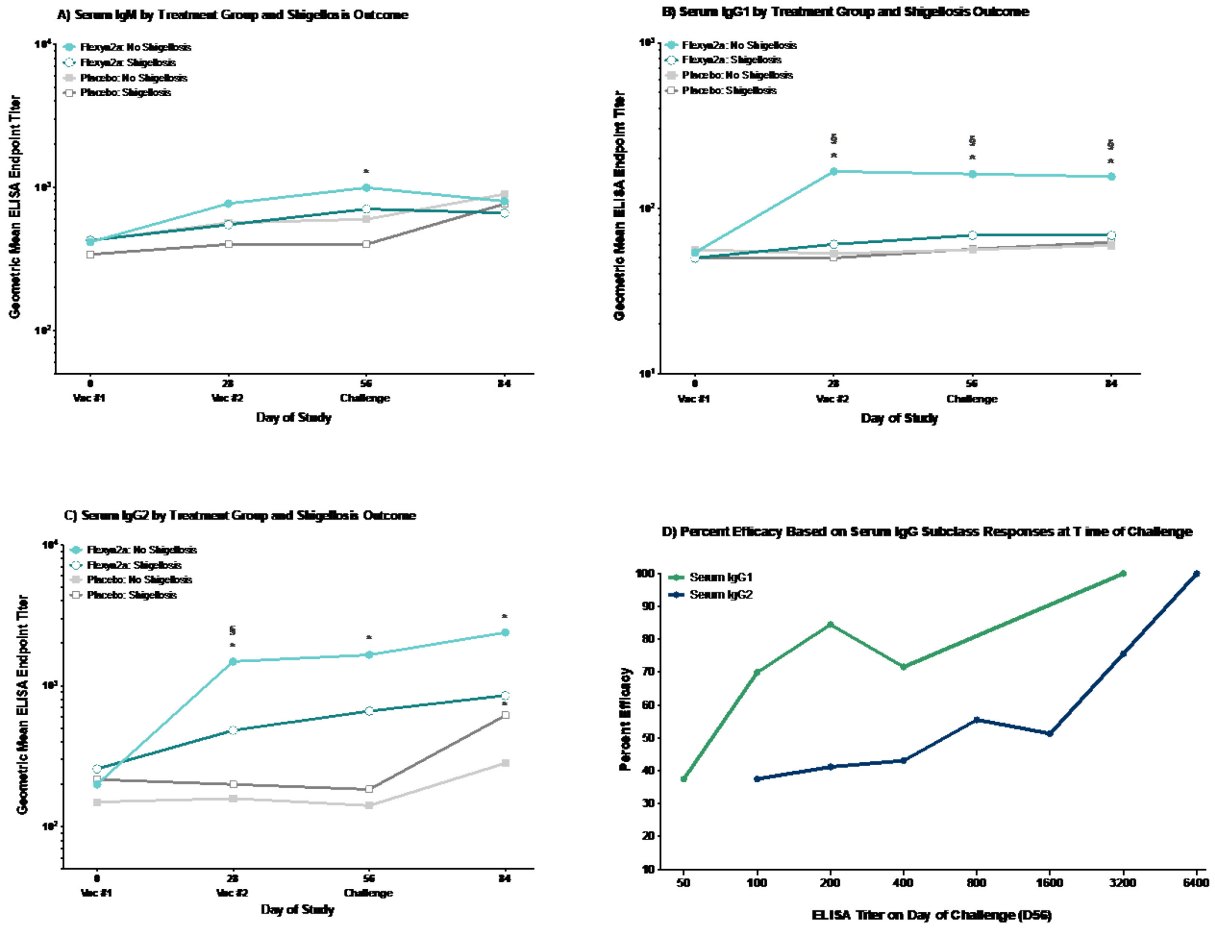
S. flexneri 2a LPS-specific serum IgG (A), IgA (B), IgG1 (C) and IgG2 (D) geometric mean ELISA endpoint titers grouped by vaccinated subjects with (N=13) or without (N=17) per-protocol shigellosis, and placebo subjects with (N=17) or without (N=12) per-protocol shigellosis. * = significant difference as compared to baseline titers within the same treatment group/shigellosis outcome. § = significant difference in titers within treatment group across shigellosis outcome at the same time point. Significance determined by 2-way ANOVA of log-transformed titers with Bonferroni post-hoc test.

Figure 4.3. *S. flexneri* 2a LPS-Specific Serum IgM and IgG Subclass Responses by Treatment Group



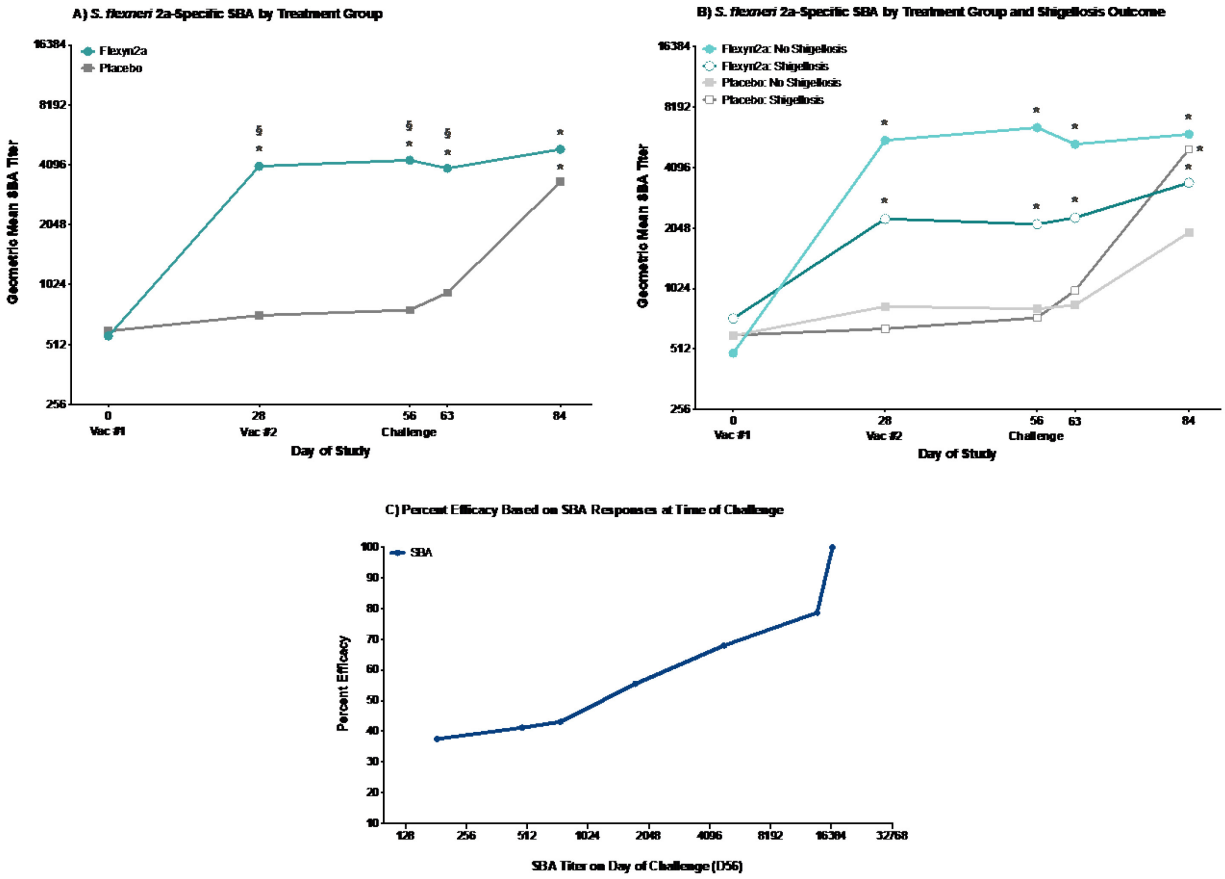
S. flexneri 2a LPS-specific serum IgM (A), IgG1 (B), IgG2 (C), IgG3 (D) and IgG4 (E) geometric mean ELISA endpoint titers grouped by vaccinated (N=30) or placebo (N=29) subjects. * = significant difference as compared to baseline titers within treatment group. § = significant difference in titers across treatment groups at the same time point. Significance determined by 2-way ANOVA of log-transformed titers with Bonferroni post-hoc test.

Figure 4.4. *S. flexneri* 2a LPS-Specific Serum IgM, IgG1 and IgG2 Responses by Shigellosis Outcome



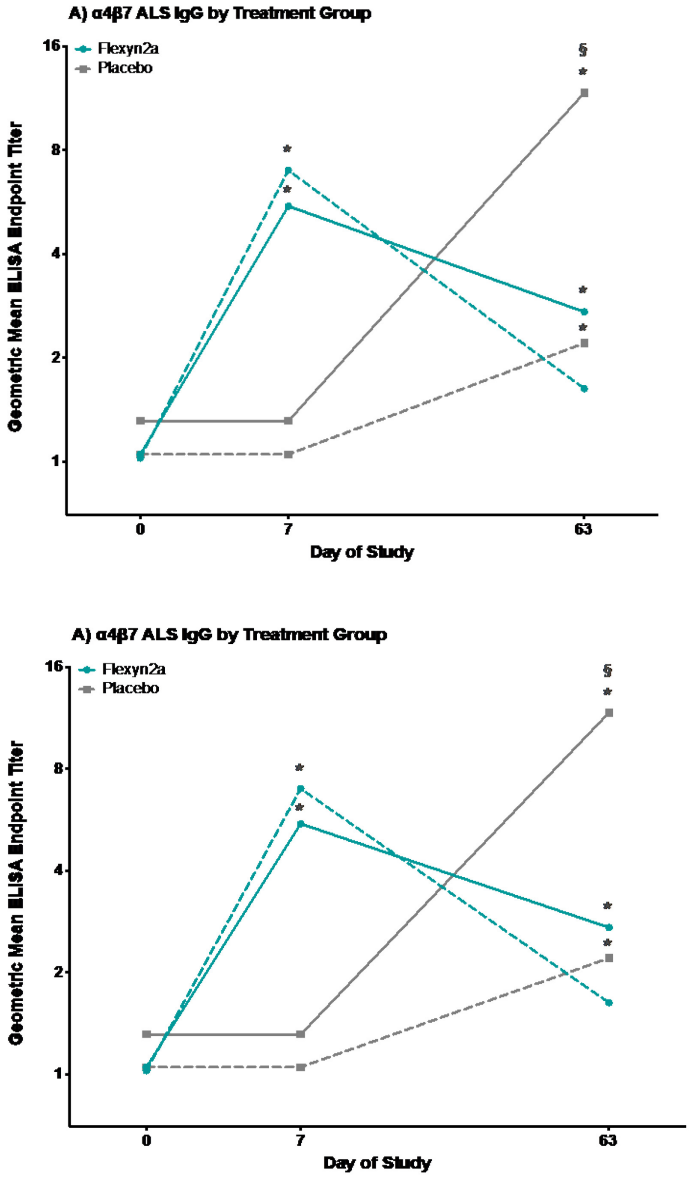
S. flexneri 2a LPS-specific serum IgM (A), IgG1 (B) and IgG2 (C) geometric mean ELISA endpoint titers grouped by vaccinated subjects with (N=11) or without (N=19) consensus shigellosis, and placebo subjects with (N=17) or without (N=12) consensus shigellosis. * = significant difference as compared to baseline titers within the same treatment group/shigellosis outcome. § = significant difference in titers within treatment group across shigellosis outcome at the same time point. Significance determined by 2-way ANOVA of log-transformed titers with Bonferroni post-hoc test. (D) Percent efficacy post-challenge in vaccinated subjects across increasing serum IgG1 and IgG2 ELISA endpoint titers.

Figure 4.5. *S. flexneri* 2a-Specific Serum Bactericidal Activity



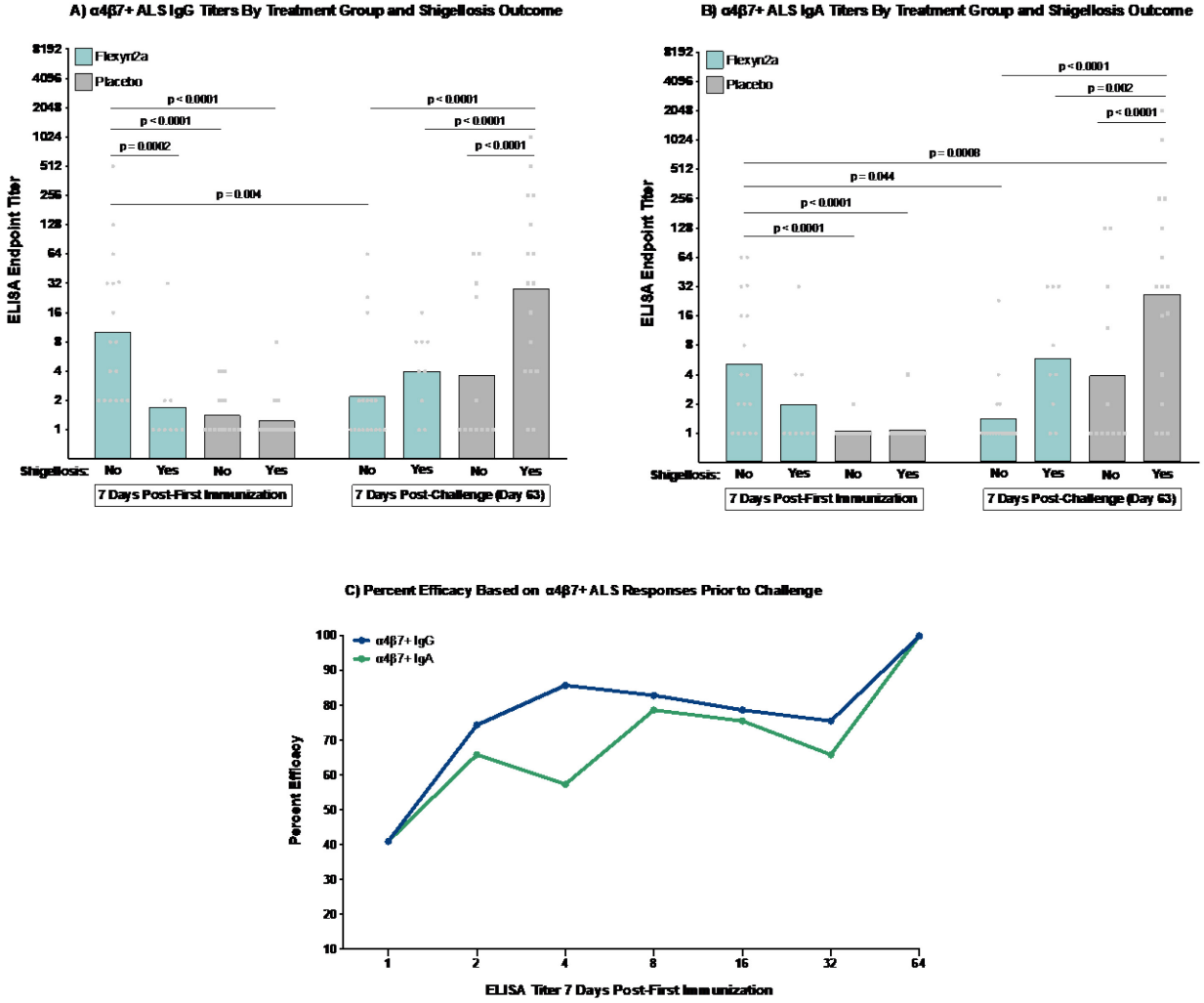
(A) *S. flexneri* 2a-specific geometric mean SBA endpoint titers grouped by vaccinated (N=30) or placebo (N=29) subjects. * = significant difference as compared to baseline titers within treatment group. § = significant difference in titers across treatment groups at the same time point. Significance determined by 2-way ANOVA of log-transformed titers with Bonferroni post-hoc test. (B) *S. flexneri* 2a-specific geometric mean SBA endpoint titers grouped by vaccinated subjects with (N=11) or without (N=19) consensus shigellosis, and placebo subjects with (N=17) or without (N=12) consensus shigellosis. * = significant difference as compared to baseline titers within the same treatment group/shigellosis outcome. § = significant difference in titers within treatment group across shigellosis outcome at the same time point. Significance determined by 2-way ANOVA of log-transformed titers with Bonferroni post-hoc test. (C) Percent efficacy post-challenge in vaccinated subjects across increasing serum bactericidal titers.

Figure 4.6. *S. flexneri* 2a LPS-Specific α4β7+ and α4β7- ALS IgG and IgA Responses by Treatment Group



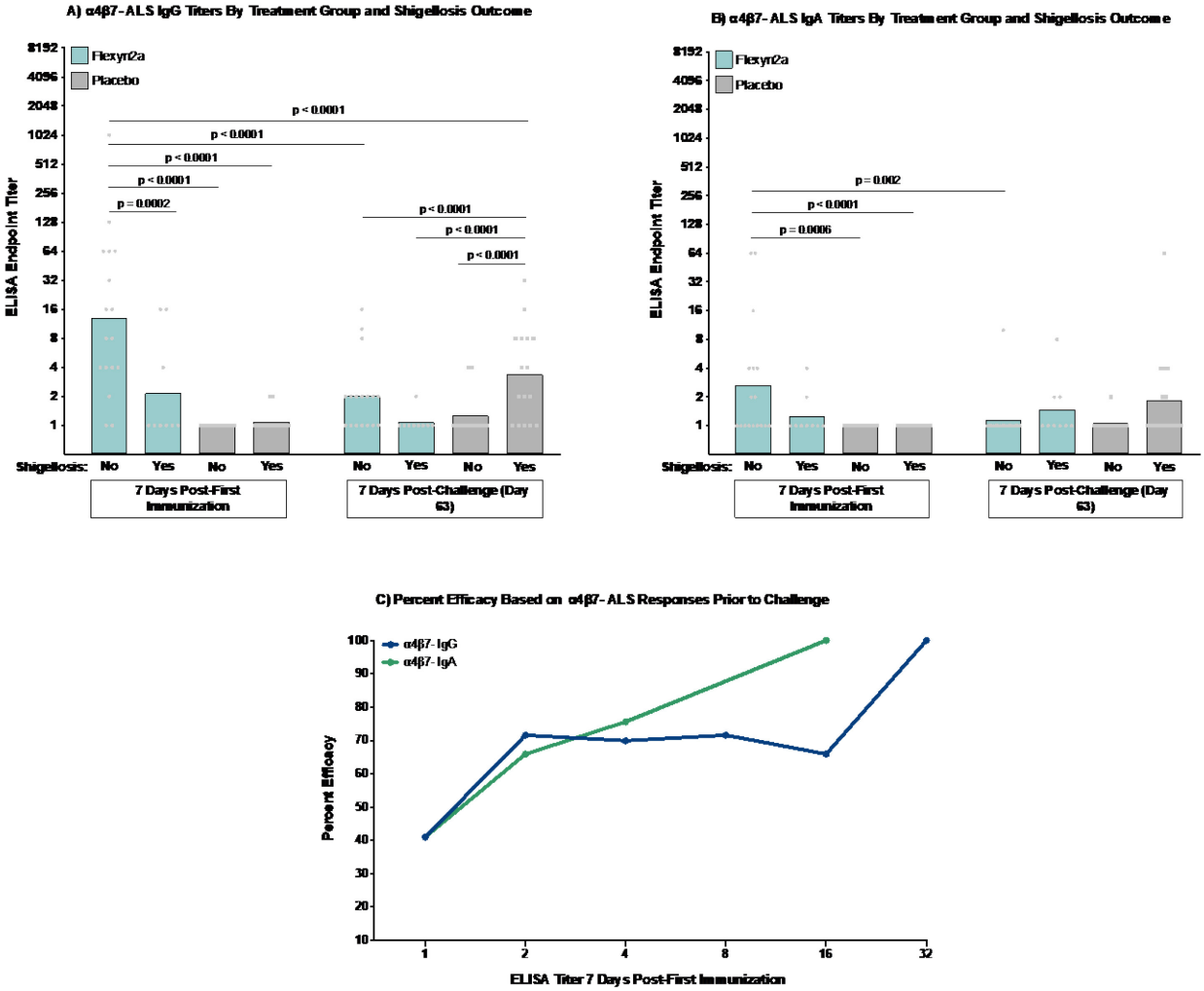
S. flexneri 2a LPS-specific ALS IgG (A) and IgA (B) geometric mean ELISA endpoint titers for α4β7+ (solid lines) and α4β7- (dashed lines) populations grouped by vaccinated (N=30) or placebo (N=29) subjects at baseline (day 0), 7 days post-first immunization (day 7) and 7 days post-challenge (day 63). * = significant difference as compared to baseline titers within α4β7 +/- population and treatment group. § = significant difference in titers between α4β7+ and α4β7- populations within treatment groups at the same time point. Significance determined by 2-way ANOVA of log-transformed titers with Bonferroni post-hoc test.

Figure 4.7. *S. flexneri* 2a LPS-Specific α4β7+ ALS IgG and IgA Responses by Shigellosis Outcome



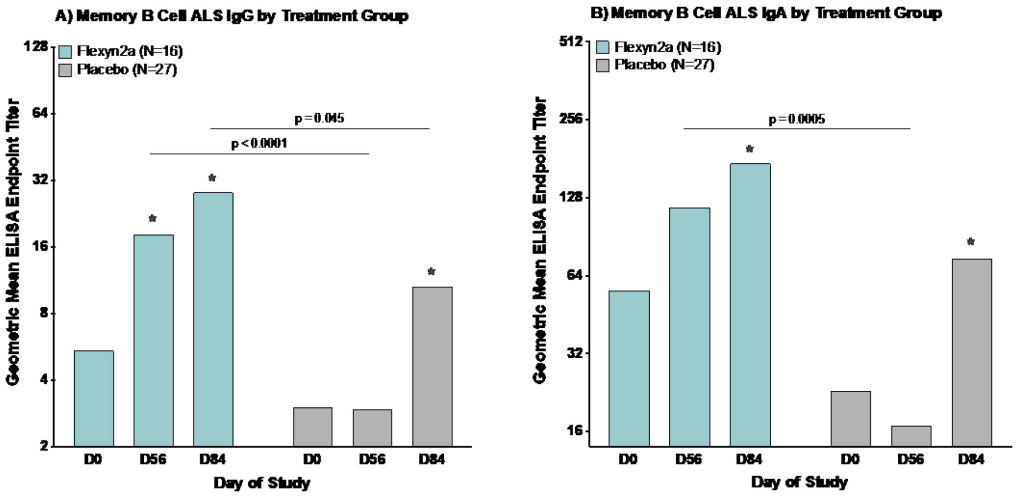
Individual *S. flexneri* 2a LPS-specific α4β7+ ALS IgG (A) and IgA (B) ELISA endpoint titers with group geometric mean either 7 days post-first immunization (day 7) or 7 days post-challenge (day 63) grouped by vaccinated subjects with (N=11) or without (N=19) consensus shigellosis, and placebo subjects with (N=17) or without (N=12) consensus shigellosis. P-values determined by 2-way ANOVA of log-transformed ALS titers with Bonferroni post-hoc test. (C) Percent efficacy post-challenge in vaccinated subjects across increasing α4β7+ ALS IgG and IgA ELISA endpoint titers.

Figure 4.8. *S. flexneri* 2a LPS-Specific α4β7- ALS IgG and IgA Responses by Shigellosis Outcome



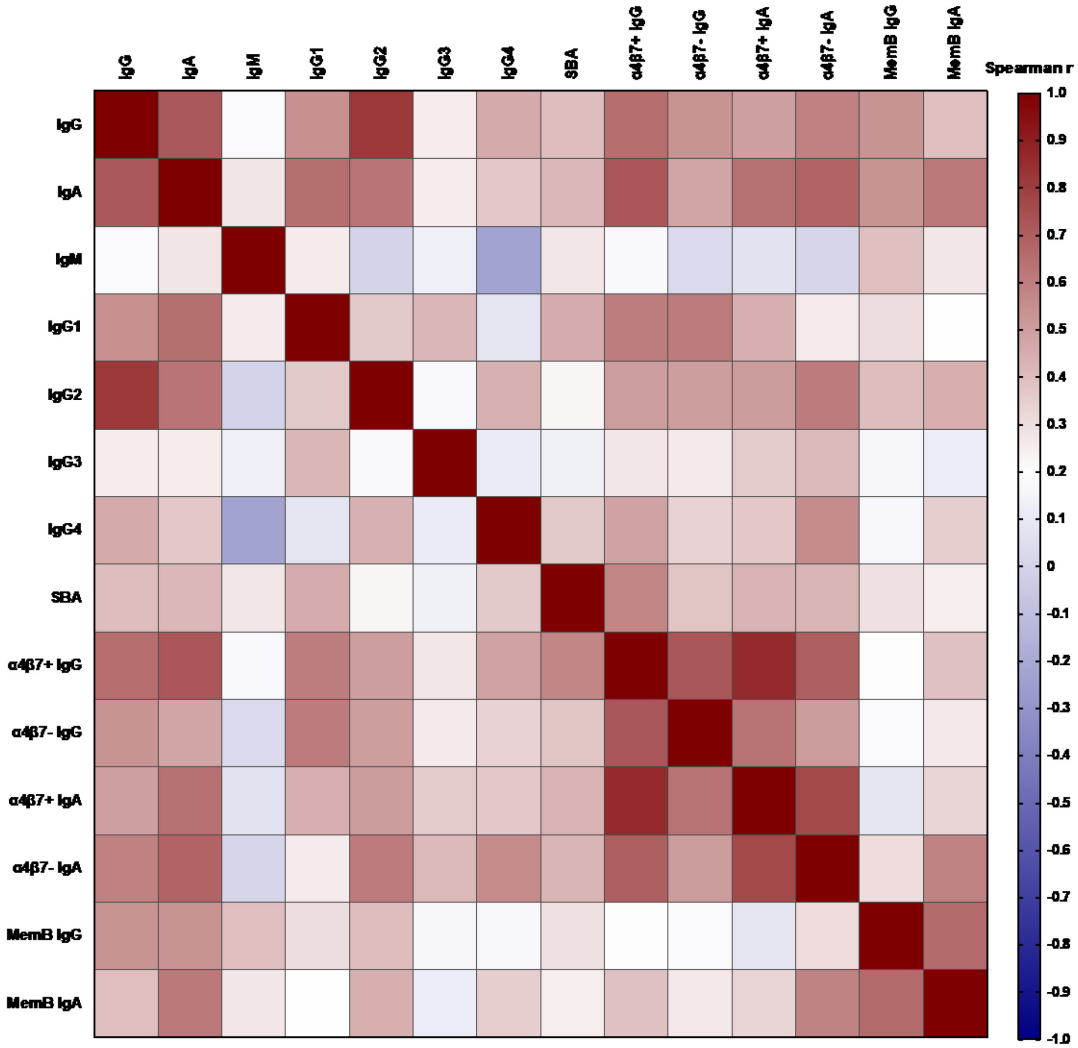
Individual *S. flexneri* 2a LPS-specific α4β7- ALS IgG (A) and IgA (B) ELISA endpoint titers with group geometric mean either 7 days post-first immunization (day 7) or 7 days post-challenge (day 63) grouped by vaccinated subjects with (N=11) or without (N=19) consensus shigellosis, and placebo subjects with (N=17) or without (N=12) consensus shigellosis. P-values determined by 2-way ANOVA of log-transformed ALS titers with Bonferroni post-hoc test. (C) Percent efficacy post-challenge in vaccinated subjects across increasing α4β7- ALS IgG and IgA ELISA endpoint titers.

Figure 4.9. *S. flexneri* 2a LPS-Specific Memory B Cell ALS IgG and IgA Responses by Treatment Group



S. flexneri 2a LPS-specific memory B cell ALS IgG (A) and IgA (B) geometric mean ELISA endpoint titers at baseline (day 0), day of challenge (day 56) and 28 days post-challenge (day 84), grouped by vaccinated (N=16) or placebo (N=27) subjects. * = significant difference as compared to baseline titers within treatment group. Lines with p-values = significant difference in titers between groups at the same time point. Significance determined by 2-way ANOVA of log-transformed titers with Bonferroni post-hoc test.

Figure 4.10. Spearman Correlation Map of Immune Response Parameters in Vaccinated Subjects



Spearman correlation heat map of immune parameters in vaccinated subjects either 7 days post-first immunization (α4β7+ and α4β7- responses) or on day of challenge/day 56 (all other immune parameters).

CHAPTER 5. *SHIGELLA*-SPECIFIC IMMUNE RESPONSE PROFILES AFTER PARENTERAL IMMUNIZATION OR ORAL CHALLENGE

5.1. ABSTRACT

Shigella species are the second leading cause of diarrhea-associated global morbidity and mortality and, while *Shigella* infection affects all age groups, children under the age of 5 years old living in LMICs are an especially vulnerable population. Although advances in infrastructure and improved access to clean water sources have reduced the *Shigella*-specific disease burden, development and widespread implementation of an efficacious vaccine remains the best option to further reduce this burden. Unfortunately, the lack of a well-defined correlate(s) of protection for shigellosis has hindered vaccine efforts thus far.

Prior *Shigella* challenge/re-challenge studies have demonstrated that infection provides protection against subsequent infection in a serotype-specific manner. These findings suggest that investigating immune responses associated with disease after *Shigella* infection may help to elucidate important mechanisms associated with protection from shigellosis. In the current analysis, two *Shigella* controlled human infection models were used investigate protective immune mechanisms and profiles associated with disease after challenge with two different *Shigella* serotypes.

Shigella-specific immune profile investigations revealed the important finding that different protective profiles were induced post-infection with *S. sonnei* as compared to *S. flexneri* 2a. This is the first report of different protective immune profiles associated with different *Shigella* serotypes, a finding which should be further investigated as it could have important implications for the *Shigella* vaccine development field.

5.2. INTRODUCTION

Shigella species are a major cause of diarrheal disease associated morbidity and mortality with approximately 75 million annual episodes of shigellosis.[1,23,27,159] Disease burden is further increased in vulnerable populations such as children less than 5 years of age living in under-developed nations.[1,23,27,159] *Shigella* infection is also associated with multiple post-infectious sequalae including reactive arthritis and irritable bowel syndrome,[20,40] as well as physical and cognitive stunting in children.[8,15] Furthermore, children with repeated enteric infections are also at a higher risk of mortality due to other infectious diseases.[17] Although antibiotics are generally effective in the treatment of shigellosis, increased rates of antibiotic-resistance require the development of alternate primary prevention methods, such as an efficacious vaccine.[55,57] While several vaccine candidates are currently under clinical investigation,[157,158] there is currently no widely available licensed vaccine to prevent shigellosis. A potential hindrance to the development of an efficacious vaccine is the lack of a defined immune correlate of protection (CoP) for *Shigella* infection.

CoPs serve as immunological parameters that are predictive of vaccine efficacy, making them vital to understanding pathogen-specific protective immunity.[78] CoPs can be mechanistic (mCoP) or non-mechanistic (nCoP), with mCoPs causally associated with protection often functioning at the site of infection. While nCoPs are not the causal means of protective immunity, they can predict protection and serve as surrogate measures for mCoPs.[78] A defined CoP can not only guide the rational design and efficacy assessments of candidate vaccines but can also be used to accelerate vaccine licensure,

especially in the case of multivalent vaccines where alterations or additions to pathogen serotypes are required.[79]

In order to investigate a CoP, the primary endpoint/outcome targeted for prevention should also be defined as it can affect the CoP.[80] The primary outcome may vary depending on the pathogen and can be as stringently defined as sterilizing immunity (i.e. preventing infection), or more loosely defined, such as preventing clinical signs and symptoms (i.e. preventing disease). Two different polio vaccines for example have different targeted levels of protection and therefore different CoPs: the live-attenuated oral polio vaccine induces strong secretory IgA responses as well as CD8+ T cell responses, working to provide protection against infection. This is in contrast to the intramuscularly delivered inactivated polio vaccine which induces robust serum antibody responses and CD4+ T cell responses and works to prevent clinical disease through the generation of high levels of serum antibodies.[84,85]

Shigella challenge/re-challenge studies have shown that prior infection protects from subsequent infection in a serotype-specific manner[98,99] demonstrating the importance of O-polysaccharide (OPS) (or O antigen, a component of the bacteria's lipopolysaccharide (LPS) molecule) as a key protective antigen. While there has yet to be a defined CoP for *Shigella* infection, LPS-specific serum IgG has been identified as a potential contender; however, discussions remain if serum IgG is acting as a mCoP via IgG transudate into the intestinal lumen or, as a nCoP or surrogate measure for a yet to be defined immunological protective mechanism.[122,123] Additional CoPs have been suggested for *Shigella* infection including LPS-specific IgA secreting B cells (ASCs),[106]

memory B cells,[129] and even a combination of a LPS-specific serum IgG and IgA ASCs.[114,128]

Investigating CoPs and developing efficacious vaccines is complicated by multiple host and environmental factors, such as population genetics or epigenetics, the target population versus the investigational population, host nutritional or immune status as well as infection history, pre-existing immunity and/or concurrent infections.[78,130-135] Each of these confounding factors is further complicated in the case of enteric pathogens due to variances in the intestinal microbiome or integrity of the gastrointestinal barrier.[133,134,136-138] Several enteric vaccines, including a rotavirus vaccine and a cholera vaccine, have demonstrated excellent success in one population followed by dramatically reduced efficacy in another population.[139-141] Furthermore, pathogen strain or serotype, route of infection and dose can not only influence pathogen virulence but also host immune response.[137,142-145] The combination of population level variances, environmental influences and differences in pathogen virulence gives rise to a dynamic interplay, not only between host and pathogen, but also within the host themselves and the immune response they mount.

Defining a CoP is often the goal in the field of vaccine development and, while there is no question regarding the value and importance of a CoP, searching for a single immune correlate may not take into consideration the complexities of the host immune response or of host-pathogen interactions. Over the past decade, advancements in the areas of systems biology and omics research have provided essential insights into host

responses to vaccination or pathogenic challenge,[130,146-150] demonstrating that protective immunity often requires an integrated and networked immune response profile.[151]

Immune response profiles post-vaccination or post-challenge have been described or proposed for several pathogens including human immunodeficiency virus, *Plasmodium falciparum* and yellow fever virus.[150,152-155] *Salmonella* Typhi-specific immune response profiles were recently investigated after parenteral immunization with two different vaccine constructs (capsular polysaccharide alone or conjugated to tetanus toxoid) followed by oral challenge with live *S*. Typhi.[156] Interestingly, while both vaccine constructs provided similar levels of efficacy post-challenge, the polysaccharide alone protected from challenge through a serum IgA dominated response while the tetanus toxoid conjugate protected subjects through increased serum IgA responses in conjunction with an increased avidity of serum IgG1 antibodies.[156]

As *Shigella* species are enteric pathogens with over 50 different serotypes (Table 2.1) and have a complex lifecycle, thorough characterization of *Shigella*-specific immunity is essential. Additionally, multiple *Shigella* vaccine constructs using different routes of mucosal or parenteral delivery are currently in development[157,158] and may provide protection from shigellosis in different ways. With no defined CoP for shigellosis and multiple host immune mechanisms that could work to reduce the severity or duration of disease, protection from shigellosis may be better described as an immune profile rather than a single immune correlate.

The analysis presented in this Chapter uses two *Shigella* controlled human infection models (CHIMs) (Appendices A and B) in order to investigate immune profiles

associated with protection from the development of shigellosis in three different sub-populations: 1) subjects parenterally immunized with a *S. flexneri* 2a bioconjugate vaccine followed by oral challenge with *S. flexneri* 2a 2457T, 2) subjects orally challenged with *S. flexneri* 2a 2457T with no prior intervention, and 3) subjects orally challenged with *S. sonnei* 53G with no prior intervention. The comparative analysis of immune profiles in these three populations holds the potential to elucidate the protective immune mechanisms associated with parenteral immunization with a *Shigella* conjugate vaccine. Additionally, this analysis could reveal immune mechanisms associated with protection after infection with two different *Shigella* serotypes, leading to discovery of similarities that could inform the prospect of heterologous protection, or differences that may be important considerations in future *Shigella* vaccine development.

5.3. MATERIALS AND METHODS

Source of Study Populations Used in Analyses. This analysis used immune response data from two *Shigella* CHIMs which are outlined in Appendices A and B. The first study is a *S. flexneri* 2a CHIM designed to assess the efficacy of a candidate bioconjugate vaccine (*S. flexneri* 2a O-polysaccharide conjugated to EPA). The study contained a vaccinated and orally challenged cohort (Figure 5.1 and Appendix B), as well as a cohort that underwent oral challenge with *S. flexneri* 2a 2457T without prior intervention (Figure 5.2 and Appendix B). Study population demographics, inclusion and exclusion criteria and disease outcomes are described in detail in Appendix B. Immune response characterization and immunoassay methodologies are described in detail in Chapter 4.

The second study was a CHIM aimed at identifying the dose of a newly manufactured lot of *S. sonnei* 53G that would induce shigellosis in ≥60% of challenged subjects (Figure 5.3 and Appendix A). Subjects in this study underwent oral challenge with increasing doses of *S. sonnei* 53G without any prior interventions. Study populations, inclusions and exclusion criteria and disease outcomes are described in detail in Appendix A. Immune response characterization and immunoassay methodologies are described in detail in Chapter 3.

Sub-Populations Used in Analyses. Three separate sample populations from the two studies outlined above were used to evaluate and compare immune response profiles after the following interventions: 1) parenteral immunization with a bioconjugate

followed by oral challenge with *S. flexneri* 2a 2457T, 2) oral challenge with *S. flexneri* 2a 2457T and 3) oral challenge with *S. sonnei* 53G.

The first sub-population (n=30) (Appendix B) was used to analyze a protective immune profile after parenteral immunization with a *S. flexneri* 2a bioconjugate vaccine (Figure 5.1).[127,204] Subjects in this sample were immunized intramuscularly twice, 28 days apart, and orally challenged 28 days after their second immunization with either 1510cfu or 1707cfu of virulent *S. flexneri* 2a 2457T. After challenge, subjects not developing shigellosis (n=19) were used to characterize protective immune profiles after parenteral immunization by investigating pre-challenge immune responses induced after vaccination.

Sample populations 2 and 3 investigate presumptively protective immune profiles post-challenge with *S. flexneri* 2a or *S. sonnei*, respectively. As prior *Shigella* infection protects from subsequent illness with the same serotype, the presumption was made that subjects progressing to shigellosis post-oral challenge would be protected from subsequent challenge with the same serotype.[98,99,177]

Sample population 2 (n=29) investigates the immune profile after oral challenge with *S. flexneri* 2a 2457T (Figure 5.2). Subjects in this sample population were orally challenged with either 1510cfu or 1707cfu of virulent *S. flexneri* 2a 2457T and post-challenge immune responses in subjects progressing to shigellosis (n=17) were used to characterize protective immune profiles associated with *S. flexneri* 2a infection.

The *S. sonnei* CHIM (Appendix A and Chapter 3) used for sample population 3 had a total of 5 cohorts (n=56) (Figure 5.3) challenged with increasing doses of virulent *S.*

sonnei 53G. Cohort 1 (n=10) was excluded from the current analyses due to the low inoculum dose (567cfu) and absence of shigellosis cases, leaving a sample population of 46 subjects used in subsequent immune profile investigations (Figure 5.3). Post-challenge immune responses in subjects progressing to shigellosis (n=22) were used to characterize presumptively protective immune profiles associated after *S. sonnei* infection.

Principal Component Analysis (PCA). PCA were used to reduce immune response data dimensionality and redundancy in order to develop new uncorrelated variables (principal components) describing the greatest amount of variability in immune response data. Principal components were used to evaluate immune response profiles across the three sample populations outlined above. All immune response data were log-transformed prior to analyses. Variable linearity and subsequent inclusion into PCA analyses was determined using a Pearson correlation matrix. Any immune response variable that did not have a Pearson r ≥0.3 with any other variable was excluded from PCA analyses. PCA sampling adequacy was assessed using Kaiser-Meyer-Olkin (KMO) measure (KMO value of ≥0.6 was required) as well as Bartlett's test of sphericity (p-value of ≤0.5 was required). After PCAs were conducted, principle component scores were assigned to all subjects for any component with an eigen value ≥1. PCA score distribution was assessed for normality by looking at distribution plots and via Shapiro-Wilk normality test with any p-value ≤0.05 indicating component scores within a population did not follow a Gaussian distribution. PCA component scores were compared using a Welch's T-Test. Non-normally distributed data were compared using appropriate non-parametric

tests. All statistical tests were interpreted in a two-tailed fashion (α=0.05) with p-values ≤0.05 considered statistically significant in Stata (Version 14 for MAC).

Immune Response Heat Maps. The highest (peak) fold-rise over baseline (day 0) (Figures 5.1-5.3) across each immune response variable was represented using heat maps in order to investigate the magnitude of immune responses across each sample population. Peak fold-rise was calculated by dividing the max titer either post-vaccination (sample 1) or post-oral challenge (samples 2 and 3) by the day 0 titer. Fold-rise in memory B cell IgG and IgA responses could not be calculated for 10 vaccinees in sample population 1 (missing value represented by grey boxes) as baseline memory B cell responses were not determined in these subjects (Chapter 4). Heat maps were generated using log-transformed peak fold-rise responses with green representing the lower end of immune response values and red representing the high end. All heat maps were generated using Prism (Version 8 for MAC).

5.4. RESULTS

5.4.1. Immune Profiles after Parenteral Immunization or Oral Challenge

Description of PCA and Statistical Results. Although *Shigella* is an enteric/mucosal pathogen, many *Shigella* vaccines currently in clinical development utilize the parenteral route of immunization with the goal of inducing potent systemic immune responses. Sample populations 1 (Figure 5.1) and 2 (Figure 5.2) were used to investigate immune profiles after parenteral immunization which were compared to the profiles induced after oral challenge with *S. flexneri* 2a 2457T. The following immune response variables had a Pearson r ≥0.3 with at least one other immune response variable and were therefore included in the PCA (data not shown): *S. flexneri* 2a LPS-specific serum IgG, IgA, IgG1, IgG2, IgG3, IgG4, memory B cell derived IgG and IgA responses, IgG and IgA secreted from B cell populations that were α4β7+ or α4β7-, and serum bactericidal activity (SBA) directed to *S. flexneri* 2a 2457T.

The peak titer post-exposure (post-vaccination or post-challenge in sample populations 1 and 2, respectively) was used for all immune response variables, with the exception of α4β7+ and α4β7- ALS responses, which were 7 days post-exposure. Immune response data from sample populations 1 and 2 were considered to have an adequate sample size for use in PCA with a KMO value of 0.761 and a significant result in the Bartlett test for sphericity ($p<0.0001$) (data not shown). Principal components 1-4 had eigen values ≥1 and explained 75.7% of the variability in immune response data across both populations. The first two components were used for comparisons across sample

populations with components (C1) and 2 (C2) accounting for 45.5% and 12.2% of the total variability, respectively (Figure 5.4A).

PCA Loading Plot. All immune response variables were positively correlated with C1, with serum IgG and IgA having the strongest correlation with C1. As all immune response variables demonstrated a positive correlation with C1, the component appears to represent the presence, or magnitude, of an immune response. On the other hand, C2 appears to differentiate the dominant phenotype of the observed immune response. Mucosal responses (as represented by α4β7+ ALS responses) are positively correlated with C2 and have the strongest correlation with this component. On the other hand, memory B cell responses, serum IgG, IgG2 and IgM are negatively correlated with C2 (Figure 5.4A), while serum IgG1 and SBA responses show minimal associations with C2. When examining the variables that have the strongest positive and negative correlations with C2, the component can be generally divided into immune response variables that define a mucosal or systemic/memory B cell immune response phenotype.

Principal Component Scores. When subject's C1 and C2 scores are plotted by exposure route (Figure 5.4B), there is an apparent differentiation in the profiles. Parenterally immunized subjects are largely divided across C1 with 62% of subjects showing a positive correlation with C1 (Table 5.1). In contrast, orally challenged subjects are divided across C2 with 67% of subjects' scores showing a positive correlation with C2 (Table 5.1). The C1 scores in parenterally immunized subjects are higher ($p=0.007$, Figure 5.4C) and show a strong positive correlation as compared to orally challenged subjects, demonstrating the robust immune response induced by parenteral immunization with

the bioconjugate vaccine as compared to the response induced after oral challenge with *S. flexneri* 2a. When examining C2 scores however, orally challenged subject scores show a stronger positive correlation with C2 as compared to parenterally immunized subjects (p=0.003, Figure 5.4C), indicating that oral challenge with *S. flexneri* 2a correlates with mucosal antibodies while responses in parenterally immunized subjects correlate better with serum antibodies and memory B cell responses (Figure 5.4).

Principal Component Scores Grouped by Shigellosis Outcome. The majority of orally challenged subjects without shigellosis demonstrate a negative correlation with both C1 and C2 (Figure 5.5A). In contrast, immune response profiles in subjects developing shigellosis are mostly positively correlated with both components (Figure 5.5A). Subjects with shigellosis show a higher magnitude of response compared to subjects without shigellosis, as represented by higher C1 scores (p=0.029, Figure 5.5B). Although not significant (p=0.071, Figure 5.5B), a similar trend is observed across C2 values with subjects progressing to shigellosis having higher mucosal responses as compared to subjects without shigellosis (Figures 5.5A and 5.5B).

Parenterally immunized subjects not protected from shigellosis demonstrate a similar profile as orally challenged subjects not progressing shigellosis, with negative C1 and C2 values (Figures 5.5C and 5.5D). In contrast, parenterally immunized subjects protected from shigellosis had robust systemic immune responses (Figure 5.6) with 77% of protected vaccinees having positive C1 values (p=0.015, Figure 5.5D). As expected, there is a high degree of protected vaccinees clustering in the lower right quadrant of the score plot with a strong positive correlation with C1 and negative correlation with C2.

The clustering of protected vaccinees observed in the lower right quadrant demonstrates the robust systemic response associated with protection from shigellosis after parenterally immunization. Interestingly, there were some protected vaccinees (35%) with positive C2 values (Table 5.2), indicating that parenteral immunization with the bioconjugate vaccine is capable of inducing a protective immune response driven by mucosal antibodies (Figures 5.5C and 5.6 and Table 5.2). Overall, the trend in immune profiles comparing parenteral and oral exposure demonstrate strong correlations (p=0.015, Figures 5.5E and 5.5F) with C1 (systemic/memory responses) in protected vaccinees while orally challenged subjects progressing to shigellosis show a stronger correlation with C2 (mucosal immune responses) (p=0.008, Figures 5.5E and 5.5F).

5.4.2. Immune Profiles after Oral Challenge with S. flexneri 2a or S. sonnei

Description of PCA and Statistical Results. As previously described, subjects progressing to shigellosis post-oral challenge were used to characterize protective immune profiles associated with S*. flexneri* 2a 2457T (sample population 2, Figure 5.2) or *S. sonnei* 53G (sample population 3, Figure 5.3) infection. The following immune response variables had a Pearson r ≥0.3 with at least one other immune response variable (data not shown) and were therefore included in the PCA: *S. flexneri* 2a LPS-specific serum IgG, IgA, IgG1, IgG2, IgG3, memory B cell IgG and IgA responses, IgG and IgA secreted from B cell populations that were α4β7+ or α4β7-, and serum bactericidal activity (SBA) directed to either *S. flexneri* 2a 2457T or *S. sonnei* Moseley. With the exception α4β7+ or α4β7- ALS, which use responses on day 7, all immune response data

is from 28 days post-challenge with either serotype. Immune response data from sample populations 2 and 3 were considered adequately sampled for use in PCA with a KMO value of 0.742 and a significant result in the Bartlett test for sphericity ($p<0.0001$) (data not shown). Principal components 1-3 had eigen values ≥1 and explained 68.3% of the variability in immune response data across both populations. The first two components were used for comparisons across sample populations 2 and 3 with C1 and C2 (C2) accounting for 43.1% and 15.9% of the total variability, respectively (Figure 5.7A).

PCA Loading Plot. Similar to the loading plot comparing parenteral versus oral exposure, all immune response variables were positively correlated with C1, indicating this component describes the immune response magnitude, with serum IgG and IgA, as well as memory B cell IgG, having the strongest positive correlations with C1. There is a high degree of clustering across C2 which also appears to describe a mucosal versus memory/systemic immune response (Figure 5.7A). Serum IgG3 as well as α4β7+ ALS IgG and IgA responses have the strongest positive correlation with C2 while serum IgG2, SBA and memory B cell IgA responses, are negatively correlated with C2 (Figure 5.7A). Serum IgG1 is the only immune response variable which is minimally associated with C2.

Principal Component Scores. Comparing subject's C1 values across challenge strain shows a wide range of values (Figure 5.7B and Table 5.3) with no differences across strain ($p=0.505$, Figure 5.7C), indicating a large amount of variability in the magnitude of the individual immune responses induced by both *Shigella* serotypes. Interestingly, component scores appear to be divided across C2 with *S. sonnei* challenged subjects having more (59%) positively correlated C2 scores ($p<0.0001$, Figure 5.7C, Table 5.3) as

compared to *S. flexneri* 2a challenged subjects. The majority (78%) of C2 values after oral challenge with *S. flexneri* 2a demonstrate negative correlations (Figures 5.7B and 5.7C and Table 5.3), indicating that *S. flexneri* 2a infection may induce a predominantly memory/systemic immune response profile as compared to a mucosal response profile.

Principal Component Scores by Shigellosis Outcome. Comparing *S. sonnei* challenged subject scores across shigellosis outcome shows no differences in C1 scores across subjects that did or did not progress to shigellosis (p=0.260, Figures 5.8A and 5.8B and Table 5.4). In contrast, subjects developing shigellosis following *S. sonnei* challenge had significantly higher C2 values (p<0.0001, Figures 5.8A and 5.8B) with the majority of subjects (77%) having positively correlated C2 values. The robust mucosal immune response profile is further evidenced when comparing the magnitude of α4β7+ ALS IgG and IgA responses in *S. sonnei* challenged subjects with and without shigellosis (Figure 5.9). Furthermore, as previously reported (Appendix A and Chapter 3), there were no differences in serum antibody responses or SBA across subjects with and without shigellosis after challenge with *S. sonnei*.

Interestingly, an opposite trend is observed in the correlation of subject scores with C1 and C2 after challenge with *S. flexneri* 2a (Figures 5.8C and 5.8D). Specifically, 80% (Table 5.4) of subjects progressing to shigellosis after challenge with *S. flexneri* 2a have higher, positively correlated C1 values as compared to subjects without shigellosis (p=0.043, Figures 5.8C and 5.8D and Table 5.4). Furthermore, *S. flexneri* 2a challenged subject scores were negatively correlated with C2, regardless of shigellosis outcome, with no differences in C2 scores across shigellosis outcome (p=0.102, Figures 5.8C and 5.8D

and Table 5.4). This observation is further evidenced by the minimal to undetectable immune responses in *S. flexneri* 2a challenged subjects not progressing to shigellosis while, in contrast, subjects progressing to shigellosis have a range of immune response magnitudes across multiple different immune parameters (Figure 5.9).

5.5. DISCUSSION

An important finding in the current study is the difference in immune profiles post-infection with either *S. sonnei* or *S. flexneri* 2a. While infection with both serotypes induced a range of immune response magnitudes (Tables 5.3 and 5.4), there is a distinct difference across the serotypes with respect to the mucosal versus systemic profile of the immune response (Figures 5.8 and 5.9). Infection with *S. sonnei* induced similar systemic and functional immune responses, irrespective of shigellosis outcome (Chapter 3); however, the difference in the mucosal responses are substantial (Figures 5.8 and 5.9) (Chapter 3). While many *S. sonnei*-challenged subjects without shigellosis also develop a mucosal response, the magnitude of response post-challenge is significantly increased ($p=0.0001$, Mann-Whitney U, Figure 5.9 and Table 5.5) in subjects progressing to shigellosis. In contrast, subjects receiving *S. flexneri* 2a who do not develop shigellosis have minimal to undetectable immune responses across the majority of parameters while subjects developing shigellosis demonstrate increases across a range of immune parameters, with the most notable differences in serum IgG, IgA, SBA and α4β7+ ALS responses (Figure 5.9 and Table 5.5). This finding suggests that subjects with moderate to severe disease after oral challenge with *S. sonnei* present with a predominantly mucosal immune response whereas oral challenge with *S. flexneri* 2a may induce a more balanced systemic and mucosal response.

Another example of the difference in the immune response profile across *Shigella* strains is in the IgG subclass responses. While LPS-specific serum IgG3 responses are observed in subjects with shigellosis post-challenge, regardless of serotype, there is a

higher proportion of serum IgG3 responders post-challenge with *S. sonnei* (15%) as compared to *S. flexneri* 2a (3.7%). Additionally, *S. flexneri* 2a LPS-specific serum IgG3 responses correlate with different immune parameters after infection across the two serotypes. Comparison of immune responses in subjects progressing to shigellosis after *S. sonnei* infection demonstrates that serum IgG3 correlates with few other parameters aside from α4β7+ ALS IgG responses (Spearman r=0.5, Figure 5.10). In contrast, serum IgG3 responses in subjects progressing to shigellosis after infection with *S. flexneri* 2a positively correlates with serotype-specific serum IgG, serum IgG1 and α4β7+ ALS IgG, (all Spearman r=0.5) (Figure 5.10). Serum IgG1 also shows a different pattern across serotypes with moderate correlations observed post-*S. flexneri* 2a infection with α4β7+ and α4β7- ALS IgG responses, serum IgG and memory B cell IgA responses (all Spearman r=0.4-0.5). On the other hand, serum IgG1 responses post-infection with *S. sonnei* show the strongest correlations with α4β7- ALS responses (Spearman r=0.5-0.6) and serum IgG2 (Spearman r=0.7) (Figure 5.10). Interestingly, while serum IgG1 is negatively correlated with serum IgG3 (Spearman r=-0.1) post-infection with *S. sonnei*, these two parameters are positively correlated post-infection with *S. flexneri* 2a (Spearman r=0.5) (Figure 5.10).

S. sonnei is a unique serotype among *Shigella* species for many reasons, with one of the more recent discoveries revealing *S. sonnei* to harbor a type 6 secretion system (T6SS).[75] T6SSs not only serve to directly kill other bacterial species, therefore playing a role in microbial competition, but T6SSs have also been associated with increased severity of host disease and prolonged survival during infection with other enteric

pathogens, including *E. coli*, *Vibrio cholerae*, and *Salmonella*.[207-211] *S. sonnei* uses its T6SS during infection to kill nearby microbial host commensals, potentially having substantial effects on the immune response induced by *S. sonnei*. Intestinal microbiota play an integral role in host immune induction and regulation with especially important roles in the large intestine. Not only can commensals in this region contribute to fitness and tolerance of regulatory T cells,[212] but they can also control the production of pro-IL-1β in order to condition resident macrophages to quickly respond to the mature active form of IL-1β.[212] Furthermore, some resident bacteria have the ability to themselves induce inflammasome-mediated secretion of IL-1β and IL-18 during an active enteric infection.[212] While the release of pro-inflammatory cytokines does increase intestinal inflammation, it also aids in recruitment of additional innate cells, such as neutrophils, to the site of infection to help clear the infection.[213,214] Killing of commensal microbiota via the T6SS of *S. sonnei* may reduce the recruitment of innate cells as well as in the inflammatory environment within the intestine.

Another unique feature of *Shigella sonnei* is the presence of an O-antigen group 4 capsule (G4C)[214,215] which, in other enteric pathogens, has been associated with increased virulence and environmental persistence.[216,217] *S. sonnei* expresses O-antigen on both core-linked LPS as well as the G4C, creating a thick layer of O-polysaccharide (OPS) on its surface.[213-215] This thick layer of OPS on the surface of *S. sonnei* reduces T3SS accessibility, thereby reducing the T3SS-dependent uptake into macrophages and subsequent vacuole escape of *Shigella sonnei*.[213,214] While the T3SS and associated virulence proteins are essential during macrophage invasion and vacuole escape *for S.*

flexneri species, *S. sonnei* has recently shown T3SS-independent uptake into macrophages.[213] The reduced T3SS-mediated cellular uptake ultimately works to further reduce the inflammatory response during *S. sonnei* infection as less cytosolic bacteria are present within host cells to contribute to the activation of caspase-1 inflammasome induced pyroptosis. Lowered caspase-1 activation would lead to reduced macrophage cell death, resulting in less release of pro-inflammatory cytokines and subsequent neutrophil recruitment.[213,214]

Although neutrophils do work to eventually control *Shigella* infection, they unfortunately contribute to damaging the epithelial cell layer during the early stages of infection. *Shigella* species make use of this damaged epithelium and inflammatory environment by manipulating the epithelial cell layer and passing through without the use of M cells.[67] Therefore, it would seem unusual for *S. sonnei* to use its unique virulence factors to reduce inflammation during infection; however, considering recent evidence suggesting that *S. sonnei* has adapted to an extracellular lifestyle,[213] a reduction in the inflammatory environment and subsequent neutrophil recruitment would contribute to prolonged survival of extracellular *S. sonnei*. In an effort to investigate the differences in inflammatory responses induced across each serotype in the current study, calprotectin and myeloperoxidase concentrations in stool samples were compared post-infection with *S. flexneri* 2a or *S. sonnei*. Both myeloperoxidase ($p<0.0001$, T-Test) and calprotectin ($p=0.0001$, T-Test) levels were significantly increased after infection with *S. flexneri* 2a as compared to *S. sonnei* (Figure 5.11) indicating that in a CHIM setting, at the

bacterial doses tested, *S. flexneri* 2a induces a higher degree of inflammation during infection.

The thick OPS layer created by the G4C and LPS of *S. sonnei* has also been shown to increase resistance to killing by serum-mediated complement activity,[214] thereby reducing the ability of the host immune system to directly kill *S. sonnei*. A reduction in complement-mediated killing would not only contribute to a further reduction in intestinal inflammation but may also contribute to an increased ability of *S. sonnei* to induce systemic shigellosis.[214,218,219] Using the rabbit ileal loop model, encapsulated *S. sonnei* have been recovered in blood and distal organs at significantly higher levels as compared to unencapsulated *S. sonnei*.[214] Furthermore, as intestinal commensal microbiota can also influence systemic immune responses,[212] a reduction in commensal bacteria through T6SS-mediated killing could further enhance the ability of *S. sonnei* to cause systemic disease.

Non-pathogen specific factors, including study design and conduct, may also explain some of the observed differences in immune profiles across serotypes as the current analysis uses populations from two separate studies conducted at different times and geographical locations. Nonetheless, both studies used similar inclusion and exclusion criteria and, while the studies do not control for genetics, epigenetics or microbiome differences, all subjects were screened for both pre-existing health conditions as well as *Shigella*-specific pre-existing immunity, resulting in a similar baseline immune status observed across the two study populations. Antigenic dose can also impact immune responses post-challenge[220,221] and it is important to consider that

subjects challenged with *S. flexneri* 2a were challenged with an approximate dose of 1600cfu while subjects challenged with *S. sonnei* were challenged with a range of doses, averaging 1147cfu across cohorts. Although the broad range of doses used for *S. sonnei* challenge should be considered, no differences in immune responses across dose groups were identified (Chapter 3 and Appendix A).

Antigenic content and route of delivery also impact the immune response post-exposure[156,220-222] as evidenced by comparisons of immune response profiles after parenteral immunization or after oral challenge with *S. flexneri* 2a. Parenteral immunization with the bioconjugate vaccine induced immune responses correlating with serum IgG, IgG2 and IgA, as well as memory B cell and functional antibody responses, while oral challenge induced a predominantly mucosal immune response. The comparison of parenteral versus oral exposure also confirmed previous findings that the bioconjugate is capable of inducing B cells secreting LPS-specific IgG and IgA which home to the intestine and are associated with protection from shigellosis (Chapter 4). While serum IgG1 responses had little correlation with the phenotype of the protective immune profile, it is interesting to note that the majority of subjects with strong serum IgG1 responses also typically had robust α4β7+ ALS IgG responses (Figure 5.6) and the two variables showed a moderate correlation with one another (Spearman r=0.5, Figure 5.10). As a PCA generates principal components which are linear combinations of correlated data sets and variables load based off how they influence or correlate with each component, the robustness of the α4β7+ ALS IgG responses may have contributed to masking the influence serum IgG1 may have on defining a protective immune profile.

Although both studies have characterized immune responses after parenteral immunization with a *Shigella* conjugate or post-oral challenge with *Shigella* species to a level not previously described, the number of immune parameters reported are relatively limited. The reporting of a limited selection of immune responses primarily focused on humoral immune responses, could have influenced or biased the conclusions drawn from current analyses. The addition of other immune parameters such a T cell responses or cytokine profiles may provide a more complete understanding of *Shigella*-specific protective immune profiles. Alternatively, a systems biology approach of investigating immune response profiles would likely provide a more refined picture of potential differences in *Shigella*-specific protective immune profiles.

Nonetheless, the evidence presented in the current study could have implications for vaccine development as the immune response required to protect from *S. flexneri* 2a infection may be different from than that required to protect from *S. sonnei* infection. While oral challenge with *S. flexneri* 2a induced a relatively balanced immune response with both systemic and mucosal antibodies associated with progression to shigellosis, this was not the case with *S. sonnei*, with the difference across shigellosis outcome being the induction and/or magnitude of the mucosal response. However, it is important to consider that post-infection protective immune profiles in the current analysis are presumptive as the subjects did not undergo a homologous re-challenge. It is also possible that the robust inflammatory responses post-infection with *S. flexneri* 2a impacted the kinetics of the immune response with the peak mucosal responses post-*S. flexneri* 2a infection missed due to the study sample collection schedule. However, while

the α4β7+ ALS responses 7 days post-infection were used in analyses, samples were also collected and analyzed 3 days post-infection with *S. flexneri* 2a with no significant increases in α4β7+ ALS IgG or IgA at the earlier timepoint.

Many vaccines currently in clinical development are *Shigella* LPS-conjugate vaccines delivered parenterally[125,127,223-225] and while the *S. flexneri* 2a bioconjugate vaccine provides an excellent example of a parenterally delivered *Shigella* vaccine being capable of inducing mucosal immune responses associated with protection, the response may not be at the magnitude required to protect from infection with *S. sonnei*. Reports of efficacy achieved after parenteral immunization with a *S. sonnei* conjugate vaccine are available,[125,223] however efficacy in these studies was age related with only adults and children ≥3 years old protected from *S. sonnei* infection. It is also important to consider that the aforementioned *S. sonnei* conjugate vaccine efficacy studies were conducted in *Shigella* endemic populations and, as the majority of *Shigella* infections occur prior to the age of 4 in such settings,[27,54] pre-existing immunity from prior infection may have contributed to the observed efficacy. While the trials did include cohorts immunized with a *S. flexneri* 2a conjugate vaccine, the *S. flexneri* 2a attack rate was too low to assess vaccine efficacy making it impossible to investigate potential differences in protective efficacy across *Shigella* serotypes using a similar vaccine construct, especially in the population of children ≤3 years old.[125,223]

The current study provides important insights into potential immunological response differences associated with infection across different *Shigella* serotypes. The differences in immune responses post-infection may be understandable when

considering the number of molecular and pathogenic differences across the two investigated serotypes reported in recent years. Nonetheless, additional investigations should be conducted, not only from other studies of parenterally administered *S. sonnei* vaccines that have undergone efficacy testing, but also from studies of live-attenuated *Shigella* vaccines. Additionally, immune responses induced post-infection in subjects living in endemic settings may provide a different immune profile altogether and should be investigated as thoroughly as possible. Thorough characterization of immune responses and immune profiles may lead to discovery of *Shigella* serotype-specific protective immune mechanisms or profiles which may guide *Shigella* vaccine development.

Table 5.1. PCA Scores: Descriptive Statistics in Subjects Parenterally Immunized or Orally Challenged with *S. flexneri* 2a

Exposure:	**Oral (*S. flexneri* 2a)**	**Parenteral**
Number of Subjects	27	26
Component 1		
N with Positive C1 Correlation (%)	8 (29.6%)	16 (61.5%)
Mean ± Stdev[a] (Range)	-0.9 ± 2.0 (-4.1 – 3.8)	0.9 ± 2.7 (-4.2 – 7.7)
95% CI[b]	-0.7 – -0.1	-0.2 – 2.0
Median (IQR[c])	-0.6 (-2.1 – 0.3)	0.2 (-0.6 – 2.9)
Component 2		
N with Positive C2 Correlation (%)	18 (66.7%)	9 (34.6%)
Mean ± Stdev[a] (Range)	0.5 ± 1.2 (-1.3 – 3.1)	-0.5 ± 1.2 (-2.6 – 1.9)
95% CI[b]	0.0 – 1.0	-1.0 – 0.0
Median (IQR[c])	0.3 (-0.6 – 1.3)	-0.9 (-1.4 – 0.8)

[a] Stdev = Standard Deviation
[b] CI = Confidence Interval of the Mean
[c] IQR = Interquartile Range

Table 5.2. PCA Scores: Descriptive Statistics in Subjects Parenterally Immunized or Orally Challenged with *S. flexneri* 2a Grouped by Shigellosis Outcome

Exposure:	**Oral (*S. flexneri* 2a)**		**Parenteral**	
Shigellosis Outcome:	**No**	**Yes**	**No**	**Yes**
Number of Subjects	12	15	17	9
Component 1				
N with Positive C1 Correlation (%)	3 (25.0%)	5 (33.3%)	13 (76.5%)	3 (33.3%)
Mean ± Stdev[a] (Range)	-1.8 ± 1.5 (-3.8 – 0.4)	-0.2 ± 2.0 (-4.1 – 3.8)	1.9 ± 2.5 (-1.7 – 7.7)	-0.8 ± 2.4 (-4.2 – 3.8)
95% CI[b]	-2.7 – 0.8	-1.3 – 0.9	0.6 – 3.2	-2.6 – 1.0
Median (IQR[c])	-2.0 (-3.0 – -0.1)	-0.5 (-1.8 – 1.5)	1.4 (0.0 – 3.9)	-0.3 (-2.8 – 0.4)
Component 2				
N with Positive C2 Correlation (%)	7 (58.3%)	5 (66.7%)	6 (35.3%)	3 (33.3%)
Mean ± Stdev[a] (Range)	0.1 ± 0.8 (-1.0 – 1.6)	0.9 ± 1.4 (-1.3 – 3.1)	-0.5 ± 1.4 (-2.6 – 1.9)	-0.5 ± 1.1 (-2.1 – 0.9)
95% CI[b]	-0.4 – 0.6	0.1 – 1.6	-1.2 – 0.2	-1.4 – 0.3
Median (IQR[c])	0.1 (-0.6 – 0.6)	1.2 (-0.6 – 2.0)	-0.9 (-1.5 – 0.8)	-1.0 (-1.3 – 0.50

[a] Stdev = Standard Deviation
[b] CI = Confidence Interval of the Mean
[c] IQR = Interquartile Range

Table 5.3. PCA Scores: Descriptive Statistics in Subjects Orally Challenged with *S. flexneri* 2a or *S. sonnei*

Challenge Strain:	***S. flexneri* 2a**	***S. sonnei***
Number of Subjects	27	46
Component 1		
N with Positive C1 Correlation (%)	15 (55.6%)	20 (43.5%)
Mean ± Stdev[a] (Range)	0.2 ± 2.1 (-3.4 – 5.4)	-0.1 ± 2.6 (-4.7 – 6.2)
95% CI[b]	-0.6 – 1.0	-0.9 – 0.6
Median (IQR[c])	0.4 (-1.2 – 1.3)	-0.5 (-1.8 – 1.1)
Component 2		
N with Positive C2 Correlation (%)	6 (22.2%)	27 (58.7%)
Mean ± Stdev[a] (Range)	-0.8 ± 1.0 (-2.7 – 1.6)	0.4 ± 1.5 (-2.5 – 4.8)
95% CI[b]	-1.2 – -0.4	0.0 – 0.9
Median (IQR[c])	-0.7 (-1.6 – -0.1)	0.3 (-0.5 – 1.1)

[a] Stdev = Standard Deviation
[b] CI = Confidence Interval of the Mean
[c] IQR = Interquartile Range

Table 5.4. PCA Scores: Descriptive Statistics in Subjects Orally Challenged with *S. flexneri* 2a or *S. sonnei* Grouped by Shigellosis Outcome

Challenge Strain:	***S. flexneri* 2a**		***S. sonnei***	
Shigellosis Outcome:	**No**	**Yes**	**No**	**Yes**
Number of Subjects	12	15	24	22
Component 1				
N with Positive C1 Correlation (%)	4 (33.3%)	12 (80.0%)	10 (41.7%)	10 (45.5%)
Mean ± Stdev[a] (Range)	-0.6 ± 1.6 (-2.8 – 1.7)	0.9 ± 2.2) (-3.4 – 5.4)	-0.5 ± 2.6 (-4.7 – 4.5)	0.3 ± 2.5 (-3.0 – 6.2)
95% CI[b]	-1.6 – 0.4	-0.3 – 2.1	-1.6 – 0.6	-0.8 – 1.4
Median (IQR[c])	-1.0 (-1.8 – 1.1)	0.7 (-0.6 – 2.6)	-0.7 (-2.7 – 1.3)	-0.1 (-1.4 – 1.2)
Component 2				
N with Positive C1 Correlation (%)	0 (0%)	6 (40.0%)	10 (41.7%)	17 (77.3%)
Mean ± Stdev[a] (Range)	-1.1 ± 0.6 (-1.8 – 0.1)	-0.5 ± 1.2 (-2.7 – 1.6)	-0.3 ± 1.0 (-2.5 – 1.6)	1.3 ± 1.4 (-0.7 – 4.8)
95% CI[b]	-1.5 – -0.7	-1.2 – 0.2	-0.8 – 0.1	0.7 – 1.9
Median (IQR[c])	-1.2 (-1.5 – -0.5)	-0.2 (-1.8 – 0.2)	-0.3 (-1.2 – 0.6)	1.1 (10^{-5} – 2.3)

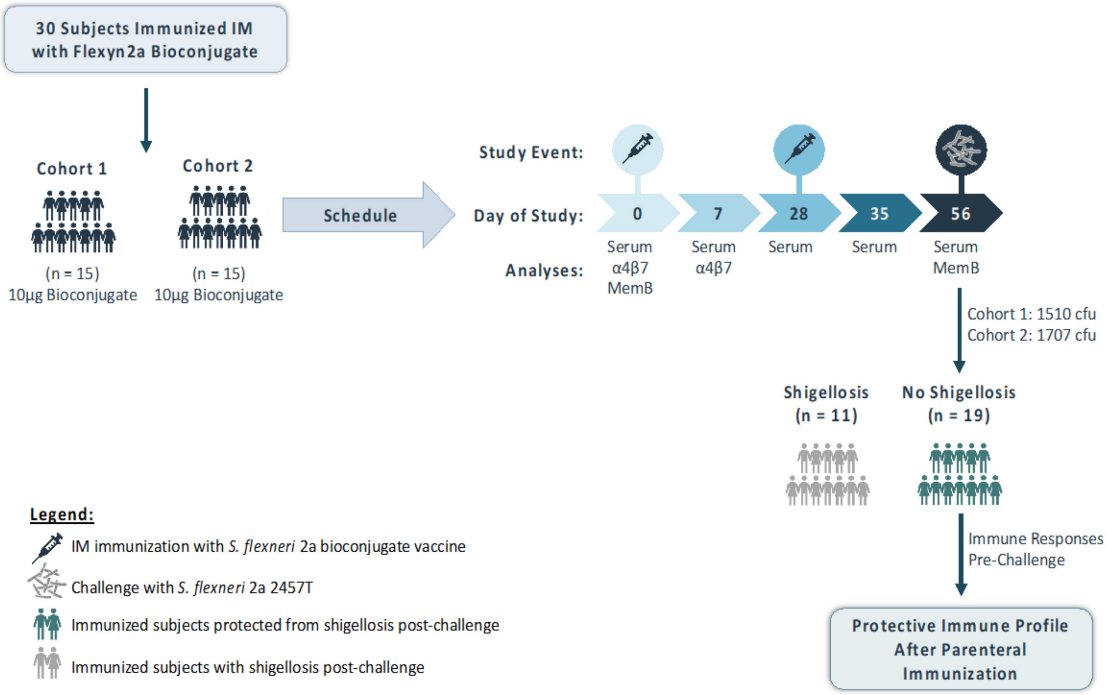
[a] Stdev = Standard Deviation
[b] CI = Confidence Interval of the Mean
[c] IQR = Interquartile Range

Table 5.5. Comparisons of Percent Responders across Shigellosis Outcome after Parenteral Immunization or Oral Challenge with either *S. flexneri* 2a or *S. sonnei*

	Percent Responders[a] and Associated P-Value								
Shigellosis:	**No**	**Yes**	**p-value[b]**	**No**	**Yes**	**p-value**	**No**	**Yes**	**p-value**
	Parenteral Immunization			**Oral Challenge (*S. flexneri* 2a)**			**Oral Challenge (*S. sonnei*)**		
Serum IgG	**100.0%**	**66.7%**	**0.004**	**33.3%**	**66.7%**	**0.020**	66.7%	100.0%	0.264
Serum IgA	100.0%	66.7%	0.177	**50.0%**	**86.7%**	**0.017**	66.7%	100.0%	0.135
Serum IgM	29.4%	11.1%	0.109	16.7%	40.0%	0.229	**41.7%**	**77.3%**	**0.042**
Serum IgG1	**58.8%**	**11.1%**	**0.022**	0.0%	6.7%	0.220	37.5%	22.7%	0.332
Serum IgG2	**88.2%**	**55.6%**	**0.013**	25.0%	53.3%	0.070	25.0%	22.7%	0.578
Serum IgG3	5.9%	0.0%	0.844	0.0%	6.7%	0.107	16.7%	13.6%	0.721
Serum IgG4	11.8%	0.0%	0.122	0.0%	0.0%	---	0.0%	0.0%	---
Bactericidal Activity	88.2%	77.8%	0.056	**16.7%**	**66.7%**	**0.026**	83.3%	90.9%	0.629
Memory B Cell IgG	66.7%	42.9%	0.120	16.7%	60.0%	0.221	54.2%	77.3%	0.347
Memory B Cell IgA	55.6%	42.9%	0.706	25.0%	60.0%	0.055	54.2%	72.7%	0.121
α4β7- IgG	**82.4%**	**33.3%**	**0.014**	**16.7%**	**46.7%**	**0.045**	**12.5%**	**36.4%**	**0.030**
α4β7- IgA	35.3%	11.1%	0.165	0.0%	20.0%	0.107	4.2%	18.2%	0.093
α4β7+ IgG	**64.7%**	**11.1%**	**0.002**	**33.3%**	**86.7%**	**0.018**	**54.2%**	**90.9%**	**0.0001**
α4β7+ IgA	52.9%	33.3%	0.133	**33.3%**	**66.7%**	**0.033**	**66.7%**	**95.5%**	**0.0001**

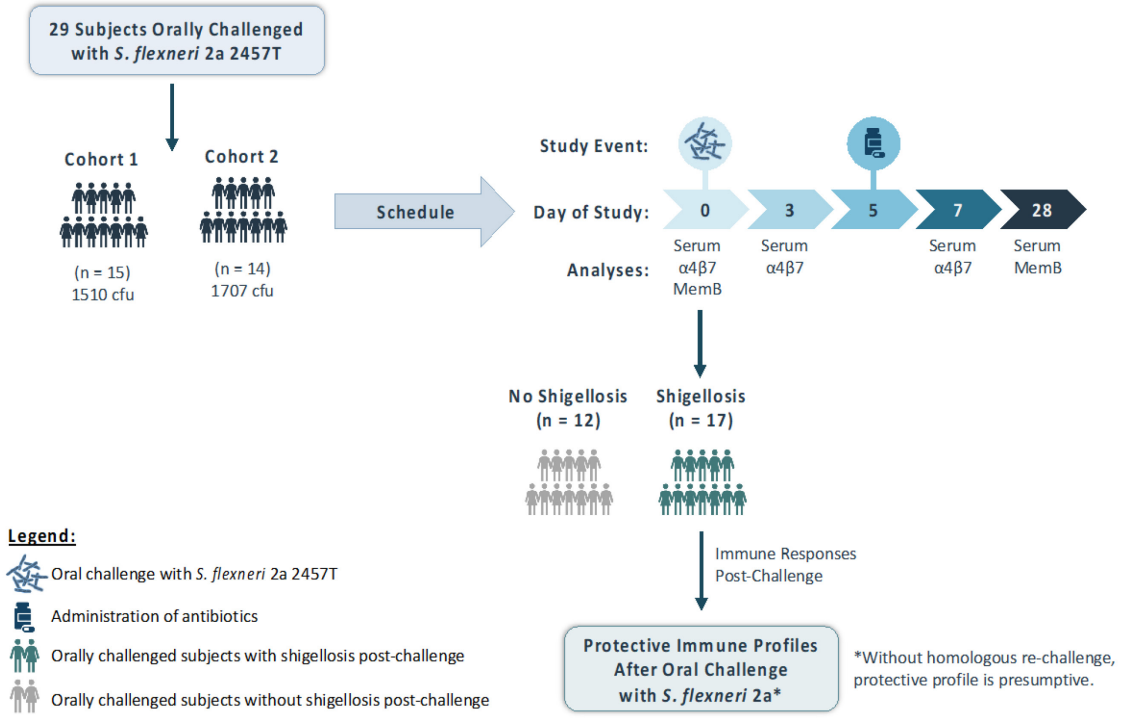
[a] Percent responder = peak fold-rise ≥4
[b] Significance determined by Mann-Whitney test comparing peak-fold rise across subjects with or without shigellosis within a population.

Figure 5.1. Sample Population 1: Subjects Parenterally Immunized with a *S. flexneri* 2a Bioconjugate Vaccine



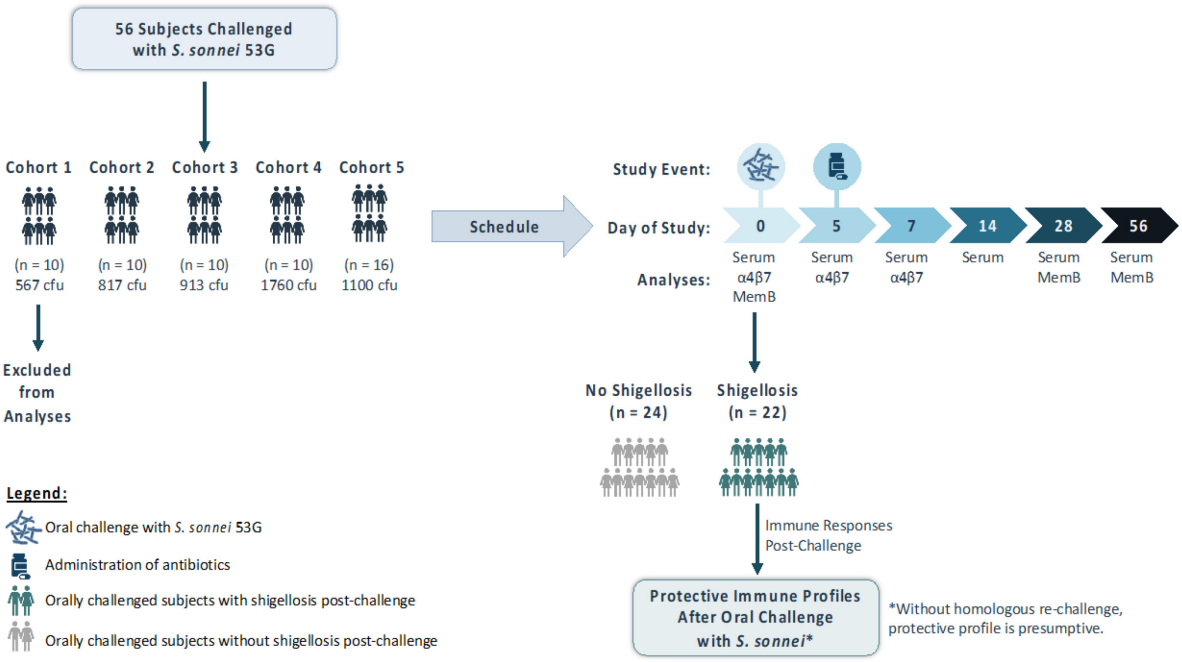
Vaccine administration and sample collection schedule in subjects parenterally immunized with a *S. flexneri* 2a bioconjugate vaccine and subsequently challenged with *S. flexneri* 2a 2457T.

Figure 5.2. Sample Population 2: Subjects Orally Challenged with *S. flexneri* 2a 2457T



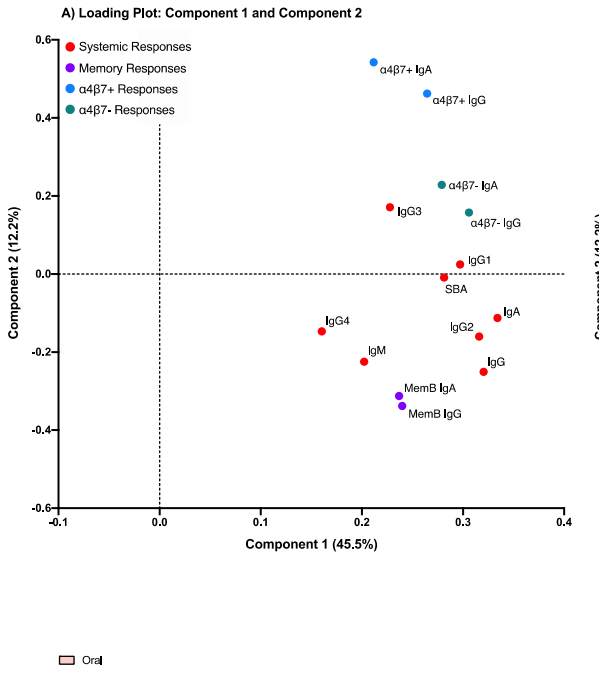
Sample collection schedule in subjects orally challenged with *S. flexneri* 2a 2457T.

Figure 5.3. Sample Population 3: Subjects Orally Challenged with *S. sonnei* 53G



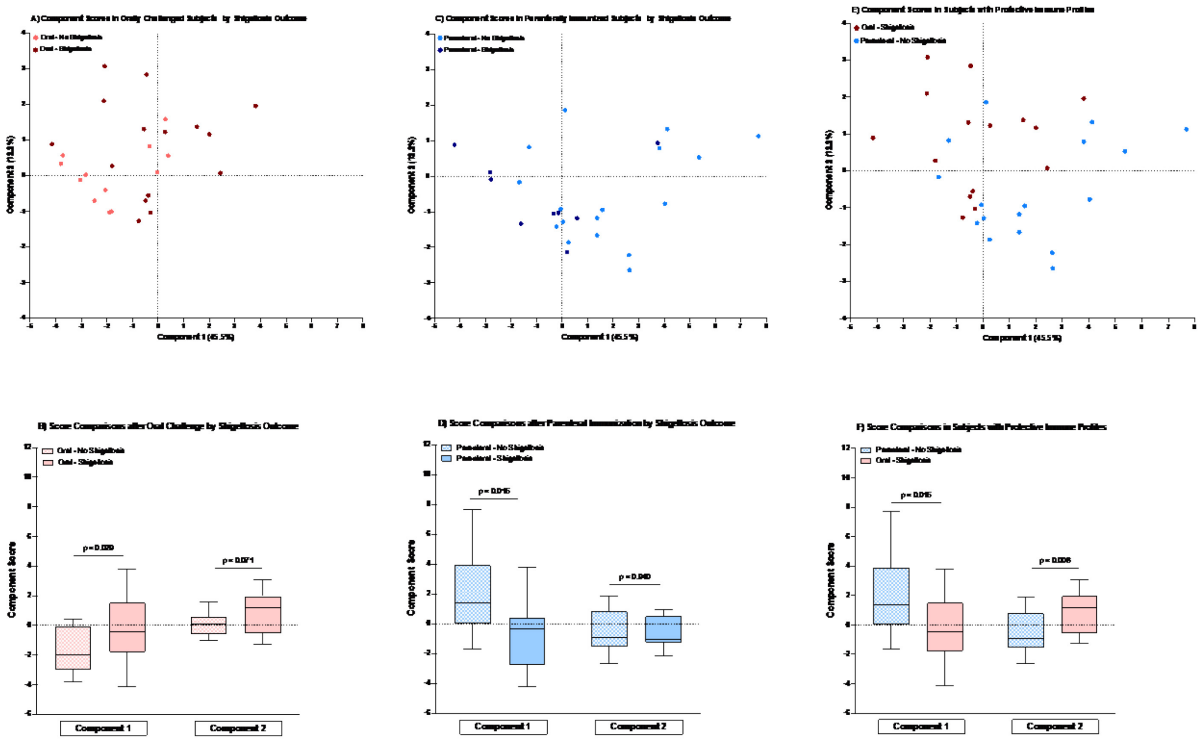
Sample collection schedule in subjects orally challenged with *S. sonnei* 53G.

Figure 5.4. Immune Profiles after Parenteral Immunization or Oral Challenge with *S. flexneri* 2a



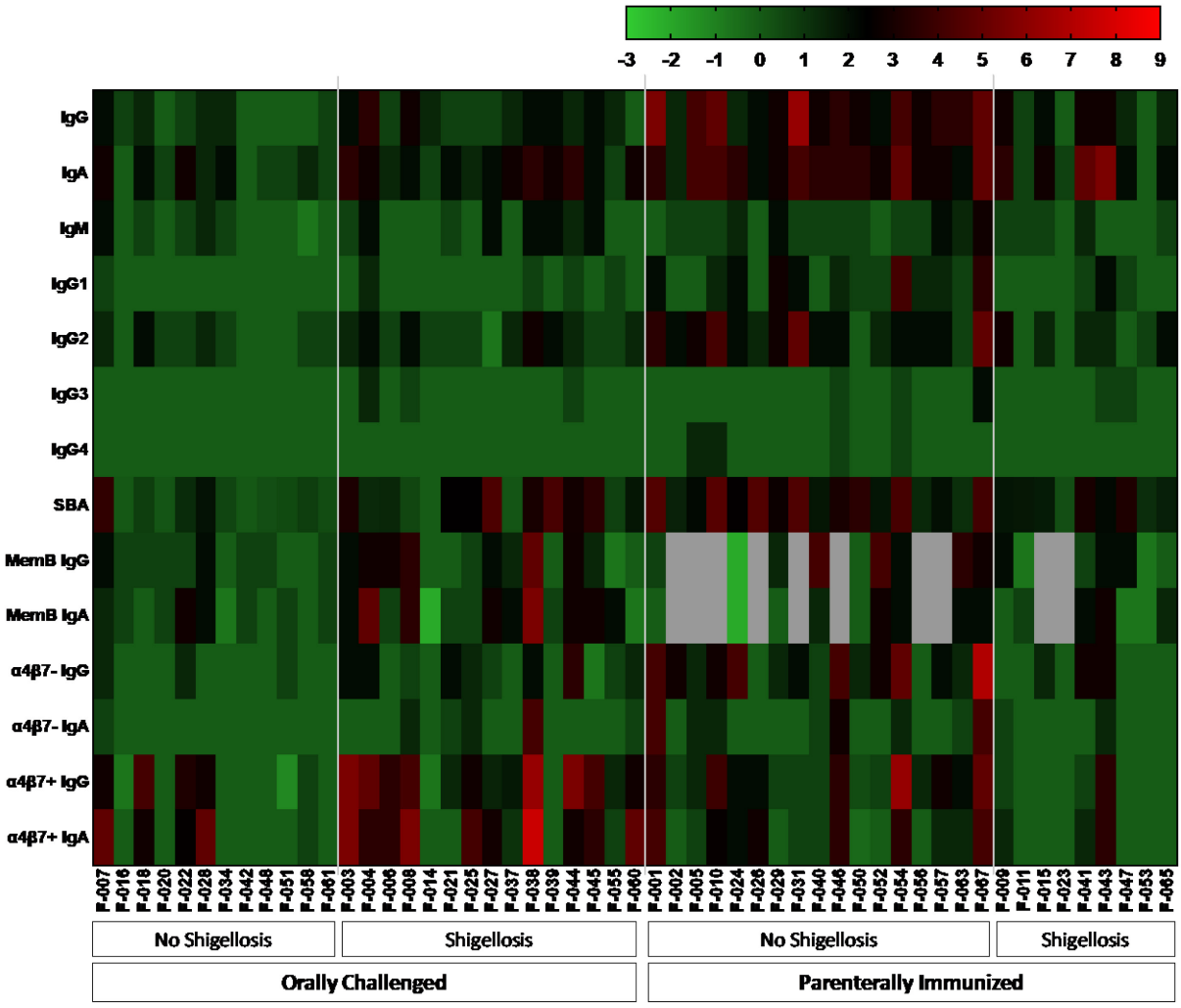
PCA results comparing Components 1 and 2 in subjects parenterally immunized with the bioconjugate vaccine or orally challenged with *S. flexneri* 2a. (A) PCA loading plot for all systemic, memory and mucosal immune response variables included in analysis, (B) Component 1 and 2 scores for all subjects used in analysis and, (C) Tukey box and whisker plots of Component 1 and 2 scores across either parenterally immunized or orally challenged subjects. P-values determined by Welch's T-test.

Figure 5.5. Immune Profiles after Parenteral Immunization or Oral Challenge with *S. flexneri* 2a Grouped by Shigellosis Outcome



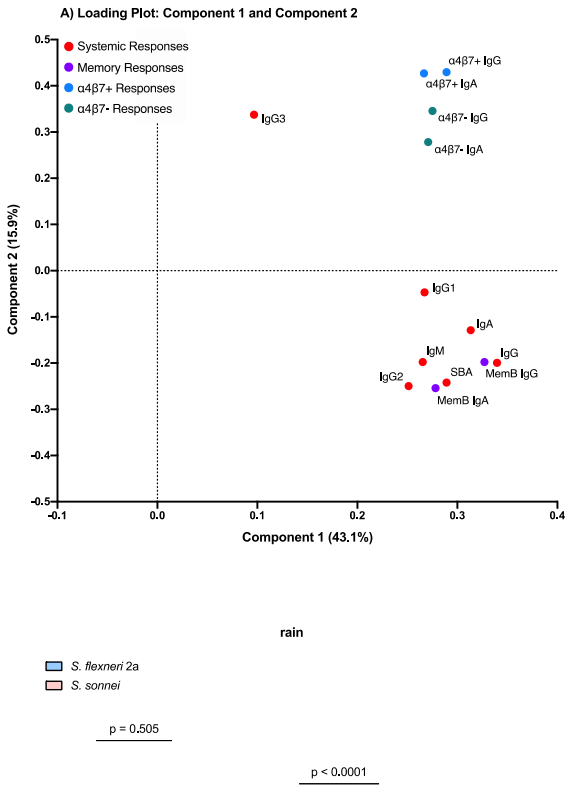
Comparisons of Component 1 and 2 scores in subjects orally challenged with *S. flexneri* 2a (A and C) or, parenterally immunized with the bioconjugate vaccine (B and D), grouped by shigellosis outcome. Tukey box and whisker plots of Component 1 and 2 scores grouped by shigellosis outcome. (E and F) Component scores and Tukey box and whisker plots in subjects either progressing to shigellosis after oral challenge or, in subjects protected from shigellosis after parenteral immunization. P-values determined by Welch's T-test.

Figure 5.6. Heat Map of Peak-Fold Rise in Immune Responses after Parenteral Immunization or Oral Challenge with *S. flexneri* 2a Grouped by Shigellosis Outcome



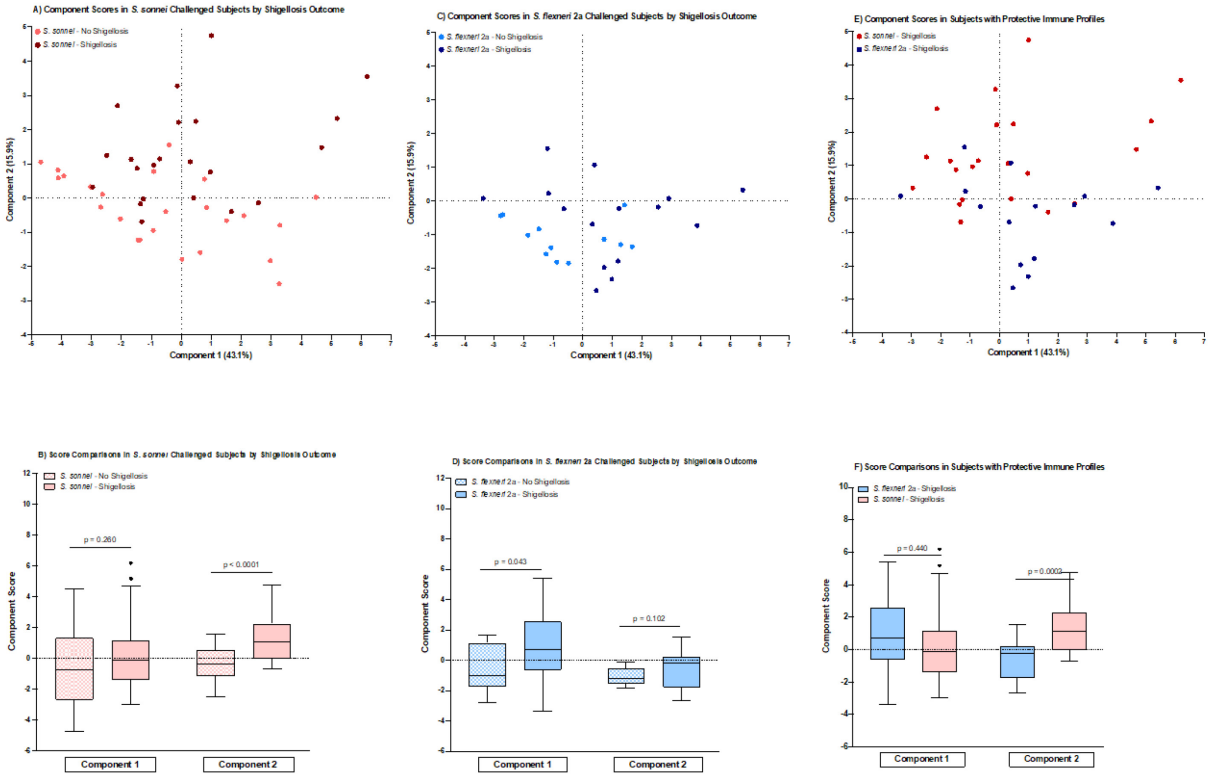
Immune response heat map comparing peak fold-rise in immune responses either post-vaccination with a bioconjugate vaccine or, post-challenge with *S. flexneri* 2a, grouped by shigellosis outcome.

Figure 5.7. Immune Profiles after Oral Challenge with *S. flexneri* 2a or *S. sonnei*



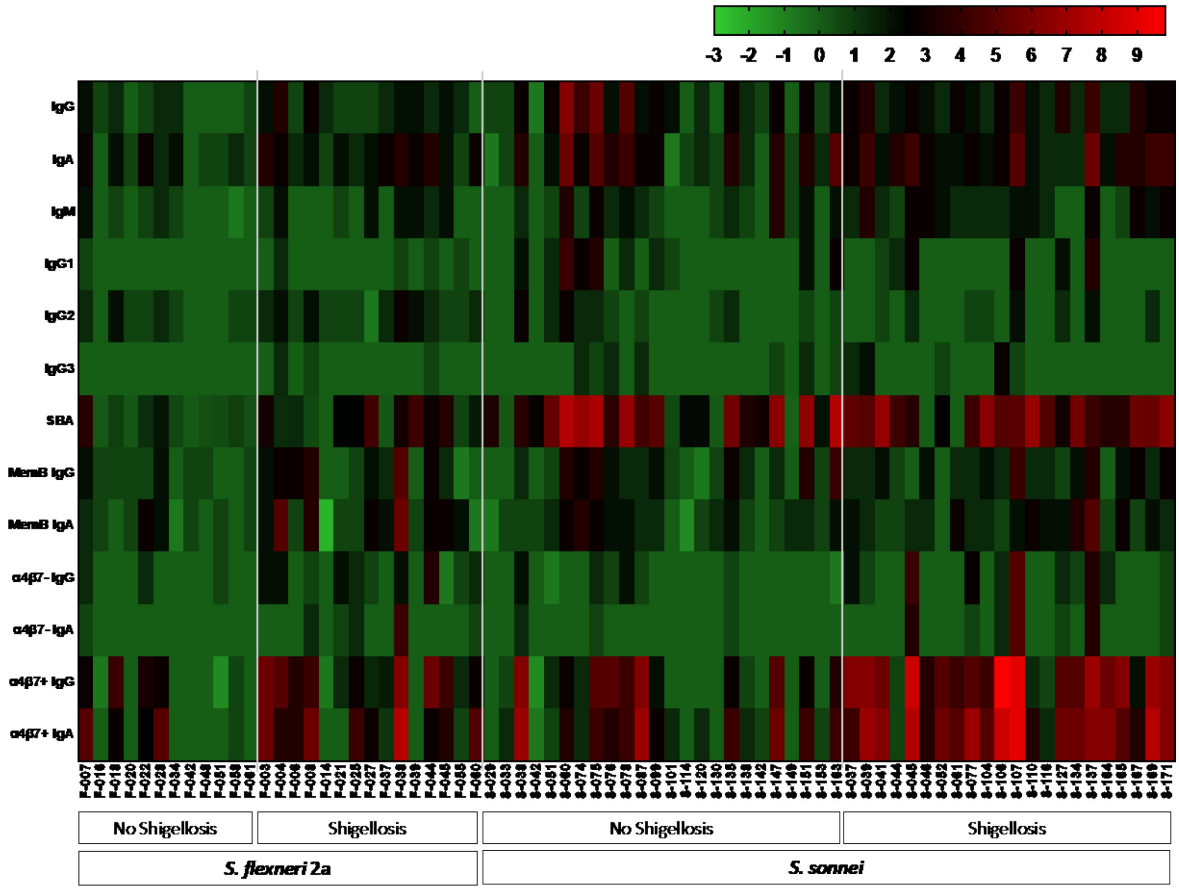
PCA results comparing Components 1 and 2 in subjects orally challenged with either *S. flexneri* 2a or *S. sonnei*. (A) PCA loading plot for all systemic, memory and mucosal immune response variables included in analysis, (B) Component 1 and 2 scores for all subjects used in analysis and, (C) Tukey box and whisker plots of Component 1 and 2 scores across subjects orally challenged with either *S. flexneri* 2a or *S. sonnei*. P-values determined by Welch's T-test.

Figure 5.8. Immune Profiles after Oral Challenge with *S. flexneri* 2a or *S. sonnei* Grouped by Shigellosis Outcome



Comparisons of Component 1 and 2 scores in subjects orally challenged with either *S. sonnei* (A and C) or, *S. flexneri* 2a (B and D), grouped by shigellosis outcome. Tukey box and whisker plots of Component 1 and 2 scores grouped by shigellosis outcome. (E and F) Component scores and Tukey box and whisker plots in subjects progressing to shigellosis after oral challenge with either *S. sonnei* or *S. flexneri* 2a. P-values determined by Welch's T-test.

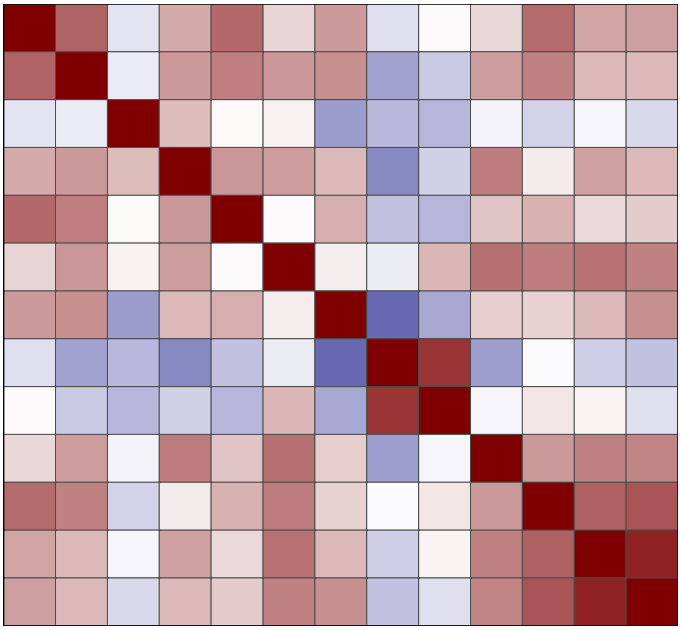
Figure 5.9. Heat Map of Peak-Fold Rise in Immune Responses after Oral Challenge with *S. flexneri* 2a or *S. sonnei* Grouped by Shigellosis Outcome



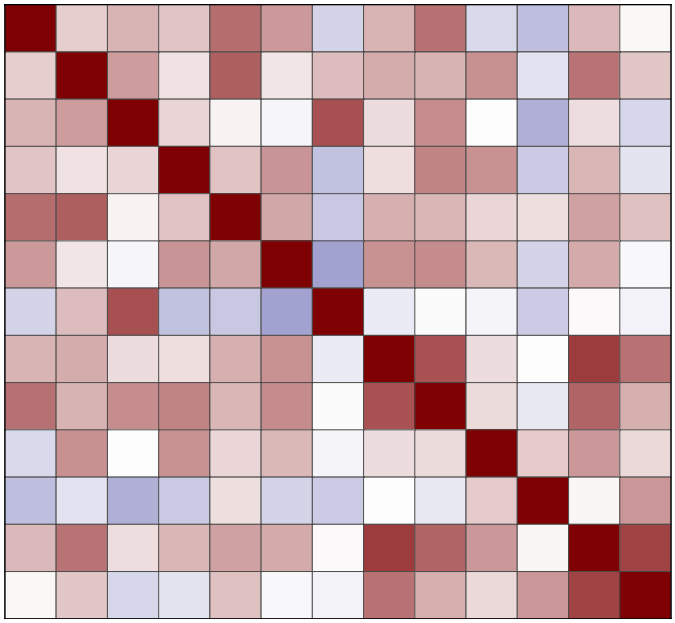
Immune response heat map comparing peak fold-rise in immune responses post-challenge with either *S. sonnei* or *S. flexneri* 2a, grouped by shigellosis outcome.

Figure 5.10. Immune Response Spearman Correlation Maps after Parenteral Immunization or Oral Challenge with *S. flexneri* 2a or *S. sonnei*

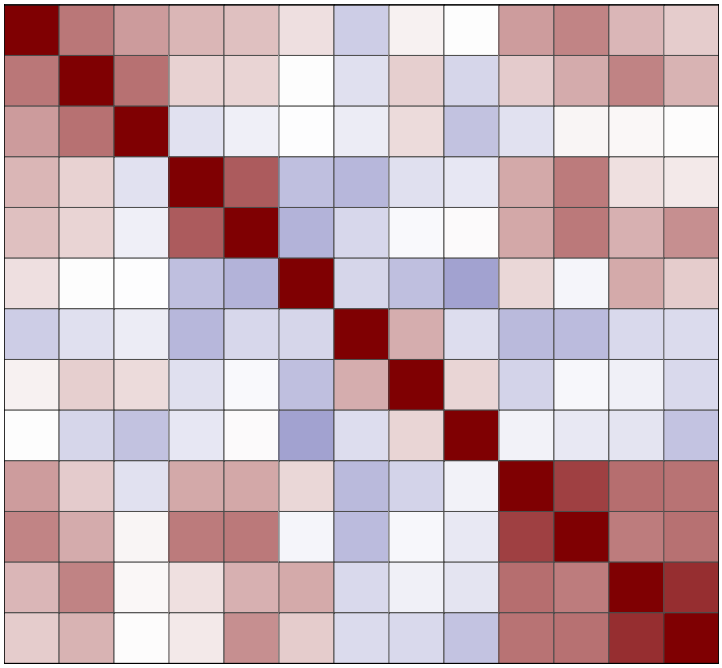
ral Challenge with *S. flexneri* 2a



***flexneri* 2a**

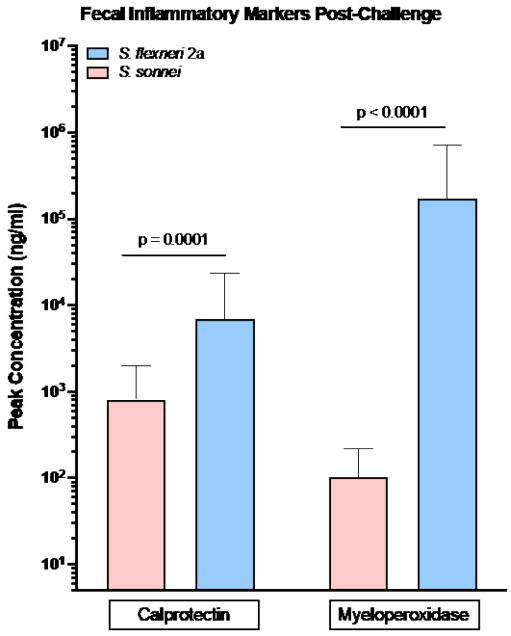


sonnei



Spearman correlation heat maps of immune response peak fold-rise in subjects (A) parenterally immunized subjects that were protected from shigellosis after oral challenge with *S. flexneri* 2a, (B) subjects progressing to shigellosis after oral challenge with *S. flexneri* 2a, or (C) subjects progressing to shigellosis after oral challenge with *S. sonnei*.

Figure 5.11. Fecal Calprotectin and Myeloperoxidase Concentrations after Oral Challenge with *S. flexneri* 2a or *S. sonnei*



Peak inflammatory marker concentrations (mean with standard deviation) post-challenge with *S. sonnei* or *S. flexneri* 2a. P-value determined by T-test of log-transformed concentrations.

CHAPTER 6. IMPLICATIONS OF CURRENT RESEARCH AND FUTURE DIRECTIONS

6.1. RESEARCH CONCLUSIONS

Major Accomplishments. The objective of the project was to characterize at a high level detail the immune responses generated in humans after parenteral immunization with a *S. flexneri* 2a bioconjugate vaccine, after oral challenge with *S. flexneri* 2a 2457T and after oral challenge with *S. sonnei* 53G. The immunological characterizations summarized within Chapters 3 and 4 have revealed important insights into immune responses associated with protection from *Shigella* infection. The insights gathered from these two chapters were then synthesized into protective immune profiles (Chapter 5) designed to inform the broader question pertaining to the development and assessment of *Shigella* vaccines.

Protective immune profiles are different based on the route of antigenic exposure (parenteral versus oral exposure). Parenteral immunization with the bioconjugate vaccine induced protection from shigellosis through multiple LPS-specific immune parameters, including serum IgG, IgG1, IgG2 and IgA. While association with protection from shigellosis was most apparent with serum antibodies, a strong association was also observed between protection and LPS-specific IgG secreting B cells homing to mucosal surfaces; a novel finding which may offer a mechanistic explanation for the protection afforded by the parenterally administered *S. flexneri* 2a bioconjugate vaccine. Although fecal IgG responses were not investigated after immunization with the bioconjugate, oral challenge with *S. sonnei* (Chapter 3) revealed a correlation between α4β7+ IgG secreting B cells and fecal IgG levels, providing indirect evidence that α4β7+ cells are homing to the intestine and contributing to intestinal IgG. It is therefore

probable that LPS-specific α4β7+ derived IgG serves as a mCoP after parenteral immunization with a *S. flexneri* 2a bioconjugate vaccine while serum IgG responses may serve as a surrogate measure or a nCoP.

Placebo subjects with moderate to severe disease after oral challenge with *S. flexneri* 2a 2457T were also used to investigate protective immune profiles. As expected, oral challenge induced a profile of robust mucosal responses accompanied by moderate systemic responses, a unique immune profile as compared to parenteral immunization characterized by robust systemic immune responses with moderate mucosal responses.

An unexpected result when comparing the two profiles was the difference in serum IgG subclass responses across the routes of antigenic exposure (Chapter 4). Moderate to robust increases in LPS-specific serum IgG2 responses were observed across all populations, regardless of exposure route or shigellosis outcome; a finding which fits dogmatic understanding of *S. flexneri* 2a-based exposures, known to induce a predominant serum IgG2 response. In contrast, serum IgG1 was the predominant IgG subclass in protected vaccinees. Furthermore, although serum IgG1 responses in protected vaccinees were lower in magnitude as compared to serum IgG2, protection afforded by the bioconjugate vaccine showed a stronger correlation with IgG1 rather than IgG2. Given that serum IgG1 responses also correlated with α4β7+ ALS IgG responses, IgG1 antibodies appear to play an important role in protection from shigellosis after parenteral immunization, potentially contributing to immunity at the site of infection. If this is the case, serum IgG1 responses could serve as an alternative surrogate measure (or nCoP) post-parenteral immunization.

Protective immune profiles are different across *Shigella* serotypes (*S. flexneri* 2a versus *S. sonnei*). Protective immune profiles were also investigated in a *S. sonnei* CHIM, facilitating comparisons of the profiles induced after oral challenge with two divergent *Shigella* serotypes. Post-challenge immune profiles revealed an unexpected difference across *Shigella* serotypes. The profile after *S. sonnei* infection showed similar systemic immune responses, regardless of disease outcome; however, subjects progressing to shigellosis had significantly higher mucosal antibody responses. Interestingly, a dissimilar pattern was observed after *S. flexneri* 2a infection with subjects progressing to shigellosis having moderate to robust increases in both systemic and mucosal responses. In contrast, the profiles in subjects without shigellosis was characterized by low to undetectable increases across both responses. Furthermore, the difference in the magnitude of the mucosal response across *Shigella* serotypes was substantial. As outlined in Chapter 5, there are several molecular and pathogenic differences between *S. sonnei* and *S. flexneri* 2a which could contribute to the observed immunological differences associated with two *Shigella* serotypes. These observations should be further explored in future studies.

In addition to comparing the immune responses post-challenge with either *S. sonnei* or *S. flexneri* 2a, the two CHIMs provided the opportunity to investigate immune responses prior to challenge and their association with a reduced risk of disease after challenge. Baseline LPS-specific serum IgA, memory B cell IgA, fecal IgA and IgG, as well as serum bactericidal activity were all associated with a reduced risk of progression to shigellosis caused by *S. sonnei*. While there were less *S. flexneri* 2a LPS-specific baseline

immune responses associated with a reduced risk of shigellosis post-challenge, a commonality was found across the two serotypes with increased baseline LPS-specific memory B cell IgA responses being associated with reduced risk of disease post-challenge. Previous reports have also shown an association of *S. flexneri* 2a LPS-specific memory B cell IgA responses with reduced disease severity after a secondary oral exposure,[129] further solidifying an essential role for LPS-specific memory IgA responses in protection against *Shigella* infection. Baseline immune responses associated with a resistance to infection have provided important information to the *Shigella* vaccine development field as potential mechanisms of protection that should be evaluated in future *Shigella* vaccine trials.

6.2. PUBLIC HEALTH IMPLICATIONS

6.2.1. Shigella Vaccine Development

Identification of key immunological mechanisms to guide the rational design of *Shigella* vaccines. Findings from the current research have important implications for future activities associated with *Shigella* vaccine development. Epidemiological data indicates that *S. sonnei*, in addition to *S. flexneri* 2a, 3a and 6, are responsible for approximately 80% of the global *Shigella* disease burden.[24,26,110] Therefore, a quadrivalent vaccine may be required to significantly reduce global disease burden, and evidence from this project indicates that different protective immune mechanisms may be required across *Shigella* species. These findings could significantly impact vaccine design and clinical testing.

If different protective immune mechanisms are required across *Shigella* serotypes, investigations into different vaccine constructs or immunization strategies may also be required in order to provide protection against the globally predominant serotypes. An alternative immunization strategy that has shown promise across other mucosal pathogens with multiple serotypes is a heterologous prime-boost regimen.[197,226-228] Priming the immune system with a vaccine consisting of one pathogen serotype and boosting with a different vaccine serotype has demonstrated increased heterologous protection across influenza, rotavirus and HIV.[229-231] For *Shigella* infection, priming the immune system with a *S. sonnei* vaccine and boosting with a multivalent *S. flexneri* vaccine (or vice versa) may induce increased protective immunity across the different *Shigella* species in order to provide the broad protection required.

Alternatively, if protection from *S. sonnei* does require a stronger mucosal immune response as compared to *S. flexneri* 2a, a prime-boost strategy investigating different vaccine constructs delivered via different administration routes could be investigated. A parenteral (or oral) priming vaccination followed by an oral (or parenteral) booster immunization has demonstrated increased efficacy and enhanced mucosal and systemic immune responses with *Heliobacter pylori*, hepatitis B and herpes simplex virus.[227,228,232] In fact, a parenteral prime/oral boost strategy has been previously investigated with *S. flexneri* in the rabbit ileal-loop model.[226] This study demonstrated a proof-of-concept for this immunization strategy as it induced increased *S. flexneri*-specific local IgA responses as well as increased systemic responses.[226] Unfortunately, as only *S. flexneri* was included in the analysis, protection provided by this strategy across other *Shigella* species could not be investigated. Nonetheless, a parenteral priming of the immune system with a *S. flexneri* multivalent conjugate vaccine followed by an oral *S. sonnei* booster immunization may induce the required protective immune mechanisms across the different *Shigella* species.

Furthermore, depending on the construct of the orally delivered *S. sonnei* vaccine, it may contain some of the virulence plasmid proteins, providing the opportunity to mount immune responses to these highly conserved antigens and potentially broaden the protection provided by such vaccine strategies. Finally, as orally delivered enteric vaccines have demonstrated reduced efficacy in children living in endemic settings and,[140,141] as the target population of a *Shigella* vaccine is children under the age of 4 living in LMICs,[27,54] priming an infant with a parenteral vaccine and boosting with an oral

vaccine when the child is slightly older may help increase oral vaccine efficacy in this population. The use of additional antigens or alternative immunization strategies certainly warrant further investigation.

Directly informing prioritization of future immunological evaluations. The field of *Shigella* vaccine development has placed a high priority on the standardization of clinical trial conduct and has not only recommended the use of consensus clinical outcome definitions but has also provided recommendations on standardized sample collection schedules and immunoassay prioritizations (Appendix C).[164] Unfortunately, these recommendations were not designed with different antigenic exposure routes or *Shigella* serotype diversity in mind. As demonstrated here, key protective immune parameters may vary, not only depending on route of exposure (parenteral or oral) but potentially, also depending on the source of antigenic exposure (*Shigella* serotype). It is therefore critical to consider immunoassay prioritization across both of these factors as they may be crucial to the evaluation of vaccines currently in development and will contribute to critical decisions regarding down selections between candidate vaccines. Furthermore, extensive immune characterizations, such as those reported in this project, may be unfeasible in many future studies due to lack of funding and potential constraints on sample collection. Therefore, information from the two *Shigella* CHIMs used in the current research was compiled in order to create immunoassays prioritizations designed to supplement the currently published recommendations (Table 6.1). While this list is not comprehensive as it only includes LPS-specific humoral immune responses, the list does

inform decisions regarding which immune parameters should be focused upon based on routes of exposure and different *Shigella* serotypes being investigated.

Immune parameters evaluated in the current CHIMs were placed into three increasing priority levels based on their association with either protection from shigellosis after parenteral immunization or, progression to shigellosis after oral challenge (Table 6.1). In addition to suggesting immunoassay prioritizations for future studies, Table 6.1 also depicts the unique immune profiles discussed in Chapter 5. For example, the prioritization list highlights the importance of LPS-specific α4β7+ and α4β7- ALS IgG responses as these measures have been identified as a primary priority, regardless of antigenic exposure and route. However, the additional importance of serum IgG, IgG1 and IgG2 responses after parenteral immunization is clear as all have also been identified as a primary priority. When assessing protection after parenteral immunization, Table 6.1 suggests that these measures could be considered when comparing different parenterally administered LPS-based *Shigella* vaccines.

When evaluating immunoassay prioritization after either *S. flexneri* 2a or *S. sonnei* oral exposure, α4β7+ ALS IgA and memory B cell derived IgA responses are listed as a primary priority, indicating that these measures may be essential when evaluating an orally delivered *Shigella* vaccine. Serum IgG, IgA and SBA responses have increased prioritization levels across both oral and parenteral *S. flexneri* 2a exposures, indicating their potential importance in protection from *S. flexneri* 2a. Interestingly, while similarities do exist across oral exposure to both *Shigella* serotypes, serum IgG stands out as being ranked the lowest priority after *S. sonnei* oral exposure while serum IgA and SBA

are a higher priority after *S. sonnei* oral exposure compared to *S. flexneri* 2a. The immunoassay prioritization list created here further emphasizes the potential immune response differences across *Shigella* serotypes.

6.2.2. Global Health

A licensed and widely available *Shigella* vaccine is a high priority for the WHO which has translated into increased attention and availability of funding.[55,56,61] The contributions provided within this research expands the current understanding of differences in protective immune mechanisms across *Shigella* species and can therefore make a positive impact on global health. If protective immunity is unique across multiple *Shigella* serotypes, different approaches to vaccine development may be required such as implementing prime/boost strategies, diversifying the antigens included in the vaccine, or utilizing adjuvants to modulate the immune response. While *S. sonnei* infections are historically associated with developed nations, there has been a shift in this paradigm with *S. sonnei* increasing in prevalence across LMICs.[18,63-65] If current vaccine strategies cannot protect against *S. sonnei* infection, but can against *S. flexneri* infections, *S. sonnei* may continue to rise in global dominance. Given the ability of *S. sonnei* to utilize a T6SS to kill commensal bacteria, increased *S. sonnei* infections in LMIC populations may further contribute to reduced intestinal barrier function and increased malnutrition in children living in LMICs. Additionally, as outlined earlier, the killing of intestinal microbiota could increase the possibility of systemic *Shigella* bacteremia, leading to serious health complications or death in an already susceptible population.

As the CDC and WHO consider antimicrobial resistant *Shigella* species to be a significant public health threat and, given the adeptness of *S. sonnei* in acquiring antimicrobial resistance (AMR) genes,[64] *S. sonnei* continuing to rise in dominance could pose a global health crisis. An increase in the prevalence of *S. sonnei* could not only provide the opportunity for lateral gene transfer of *S. sonnei* AMR genes to other bacterial species but could also allow the opportunity for *S. sonnei* to acquire additional AMR genes, further reducing available treatment options for *S. sonnei* infection. The seriousness of this threat could be further exacerbated in the context of infection with *S. dysenteriae* 1, the *Shigella* serotype typically associated with epidemics and known to harbor the deadly Shiga toxins. Should an outbreak of *S. dysenteriae* 1 occur in an area where *S. sonnei* has become endemic, it could potentially result in *S. dysenteriae* 1 acquiring AMR genes from *S. sonnei* or, alternatively, *S. sonnei* acquiring the genes encoding for the Shiga toxins from *S. dysenteriae* 1, as previously reported with *S. flexneri* species.[73,74] Either outcome could have significant impacts on global public health.

With greater than 50 known *Shigella* serotypes,[62] it is also possible that once the burden of the currently relevant serotypes is successfully reduced, other serotypes may fill the void in global dominance, a phenomenon termed serotype replacement.[233] This process of serotype replacement has been well documented in the case of *Streptococcus pneumoniae* after the release of an efficacious 7-valent vaccine.[233,234] Although this vaccine significantly reduced the disease burden caused by the globally relevant *S. pneumoniae* serotypes, within 5 years other serotypes not contained within the 7-valent

formulation had increased in global prevalence, bringing disease burden levels back to what was observed prior to vaccine implementation.[233,234]

While extensive molecular characterizations have been performed on current globally relevant *Shigella* serotypes, it is important to consider that other *Shigella* serotypes have likely not undergone such extensive characterization testing. Additionally, given the potential for different protective immune mechanisms associated with different *Shigella* serotypes, it is essential to continue characterizing protective immunity across multiple *Shigella* serotypes. Understanding differences in molecular pathogenesis or protective immune mechanisms could help prepare the global health community for epidemic changes in *Shigella* serotypes.

6.3. PROJECT LIMITATIONS

While the molecular differences between the two *Shigella* serotypes could provide some explanation into the different immune response profiles induced post-challenge, the two CHIMs used to define protective immune profiles in the current analyses were not originally designed or powered for this purpose. Although the studies used similar inclusion and exclusion criteria (Appendices A and B), the populations are nonetheless different with *S. flexneri* 2a challenged subjects recruited from the Baltimore MD area and *S. sonnei* challenged subjects recruited from the Cincinnati OH area. While both studies were conducted in North American adults, there are substantial genetic, epigenetic and microbiomic differences even within similar populations. Extrapolation of the current findings into populations from other geographical areas with potentially diverse genetic backgrounds becomes increasingly complex. Furthermore, while *Shigella* CHIMs are extremely useful tools for immunological investigations and for initial assessments of vaccine efficacy, North American adults are quite different compared to the target population of young children living in *Shigella* endemic settings. As previously mentioned, children in such settings add the additional complications of different immune system maturity as well as unknown or variable nutritional and immune status, each of which contribute to differences in immune and vaccine responses.

The studies used in the current investigations were also designed and powered to answer a different primary outcome than defining immune response profiles. While the number of subjects and immune responses investigated met the statistical requirements for PCA, such investigations would be best conducted on a larger number of subjects

enrolled into a study better designed to answer the question of differences in immune profiles post-infection across serotypes. Additionally, it is important to understand that post-infection protective immune profiles described here are presumptive. While this presumption is based on a breadth of studies demonstrating that subjects progressing to disease post-infection with shigellae are protected from subsequent disease caused by the same serotype,[98,99,109,177] the described immune profiles cannot be defined as protective without all subjects undergoing a secondary homologous challenge in order to fully assess immunological protection.

Furthermore, although both studies have also characterized the immune responses post-challenge with *S. sonnei* and *S. flexneri* 2a to a level not previously reported, the selected immune parameters are nonetheless limited and are only a representation of humoral immune responses. The investigation of limited humoral immune responses could have introduced bias and influenced conclusions. The addition of cellular immune responses or cytokine profiles would be important, especially given the potential differences in extracellular and intracellular life-cycle stages across *S. sonnei* and *S. flexneri* 2a. Additionally, further characterization of humoral immune responses such as investigating Fc glycosylation patterns and antibody affinity and avidity, or next generation analyses such as systems serology could help refine the immune profiles described here and reduce the potential of biased conclusions.

Immune responses to additional antigens, including the highly conserved Ipa proteins, should also be conducted to investigate the protective capacity of these antigens, or their ability to provide heterologous protection across *Shigella* serotypes or

species.[111,113] Serum samples from the *S. sonnei* 53G CHIM have been analyzed in a recently developed *Shigella* protein and LPS microarray[235] and results indicate a potential role for *Shigella* proteins in protection from shigellosis. One protein of particular interest is the highly immunogenic IpaB protein. Microarray analyses showed that increased IpaB-specific serum antibody responses at baseline are associated with a reduced risk of disease post-challenge (Figure 6.2A); a result mirrored by ELISA titers generated from the study (Figure 6.2B). Furthermore, additional analyses of microarray results demonstrated that subjects with increased baseline serum responses to both *S. sonnei* LPS and IpaB have a reduced risk of progression to shigellosis post-challenge (Figure 6.3). The microarray data demonstrates the importance of additional *Shigella* antigens and their potential role in protection from disease and therefore in *Shigella* vaccine development.

6.4. FUTURE RESEARCH AND NEXT STEPS

Fortunately, there are several upcoming or ongoing clinical investigations which may provide the opportunity to further elucidate or refine *Shigella*-specific protective immune profiles. In addition to the serum samples from the *S. sonnei* 53G CHIM, α4β7+ and α4β7- ALS samples have also been analyzed in *Shigella*-specific antigen microarrays which could provide important information regarding the antigen-specific mucosal responses. Initial results from these analyses indicate the potential of cross reactivity of LPS purified from *S. sonnei* and *S. flexneri* 6 (data not shown); however, results are preliminary and require further investigation into potential explanations for the observed cross-reactivity. There are also many other preserved samples from the *S. sonnei* CHIM which are planned for use in additional immunological analyses, including systems serology and microbiome analyses. While these samples are available from the *S. sonnei* CHIM, similar samples are not available from subjects in *S. flexneri* 2a CHIM. Additionally, in order to truly define protective profiles, a broader systems biology approach to investigating immune responses after a secondary homologous challenge could potentially be required.

Immune profile investigations using samples or data collected from additional retrospective CHIM studies could also help define *Shigella*-specific protective immunity. Several *S. sonnei* and *S. flexneri* 2a vaccine efficacy CHIMs have been conducted over the years which have investigated many vaccine types and administration routes, including oral live-attenuated or killed whole cell vaccines, intranasal subunit vaccines and, parenterally delivered conjugate or subunit vaccines. Immune response characterizations

using samples from these studies could provide information on the differences between *S. sonnei* and *S. flexneri* 2a protective immunity after different routes of exposure. One drawback of retrospective studies is that not all studies used similar sample collection schedules or disease outcome definitions, as recommended by the recent *Shigella* CHIM consensus papers.[164-167] It is essential to incorporate the consensus information in future studies, as well as to focus on immunoassay standardization, in order to reduce the variability in CHIM results achieved across different institutions and therefore allow more accurate comparisons of immune responses and vaccine efficacy.

Several *Shigella* CHIMs have been recently funded and will also provide the opportunity to further characterize protective immune mechanisms. The Johns Hopkins University will conduct a heterologous re-challenge study with *S. sonnei* and *S. flexneri* 2a. Subjects in this study will undergo a primary challenge with either *S. sonnei* 53G or *S. flexneri* 2a 2457T. After recovery, a subset of subjects challenged with each serotype will undergo a heterologous re-challenge in order to assess the potential cross-protection provided by the two *Shigella* serotypes. Sample collection schedules and disease outcome definitions to be used in this study have been aligned with the consensus reports in order to allow comparability across recently generated data. This study provides an excellent opportunity to not only further investigate the different *S. sonnei* and *S. flexneri* 2a immune profiles presented here but also to assess whether the profiles associated with each serotype could provide heterologous protection.

Finally, the exciting opportunity to establish the *S. sonnei* 53G CHIM in an endemic setting has also recently been funded. The same *S. sonnei* 53G lyophilized strain

will be used in a Kenyan adult population to determine the dose of *S. sonnei* that will induce a ≥60% shigellosis attack rate in a *Shigella* endemic population. As this study will follow an identical design to the *S. sonnei* CHIM conducted in North American adults (described in Chapter 3 and Appendix A), it will provide essential information regarding the bacterial dose required to achieve the desired attack rate, as well as immune responses pre- and post-infection in a population likely to have pre-existing *Shigella*-specific immunity. This will also be the first time a *Shigella* CHIM would be conducted in an African LMIC, providing the opportunity to establish a model where *Shigella*-specific interventions can be tested in the adult subset of the target population. Data presented here contributes to both the *Shigella*-specific fields of vaccinology and immunology and can also help guide the design and conduct of upcoming *Shigella* CHIMs in order to ultimately better inform vaccine development investments as the field looks to reduce the global morbidity and mortality associated with *Shigella* infections.

Table 6.1. *Shigella* CHIM Assay Prioritization List after Parenteral Immunization or Oral Challenge with *S. flexneri* 2a or *S. sonnei*

Antigenic Exposure (Route)	Systemic Compartment	Mucosal Compartment	Memory and Functional Compartment
***S. flexneri* 2a (Parenteral)**	IgG	α4β7+ ALS IgG	Memory B Cell ALS IgG
	IgG1	α4β7- ALS IgG	Memory B Cell ALS IgA
	IgG2	α4β7+ ALS IgA	Bactericidal Activity
	IgG3	α4β7- ALS IgA	
	IgG4		
	IgA		
	IgM		
***S. flexneri* 2a (Oral)**	IgG	α4β7+ ALS IgG	Memory B Cell ALS IgG
	IgG1	α4β7- ALS gG	Memory B Cell ALS IgA
	IgG2	α4β7+ ALS IgA	Bactericidal Activity
	IgG3	α4β7- ALS IgA	
	IgG4		
	IgA		
	IgM		
***S. sonnei* (Oral)**	IgG	α4β7+ ALS IgG	Memory B Cell ALS IgG
	IgG1	α4β7- ALS IgG	Memory B Cell ALS IgA
	IgG2	α4β7+ ALS IgA	Bactericidal Activity
	IgG3	α4β7- ALS IgA	
	IgG4		
	IgA		
	IgM		

Pre-challenge (parenteral) or post-challenge (oral) immune response parameters were prioritized by examining significant differences ($p \leq 0.05$) across shigellosis outcome in the following measures:

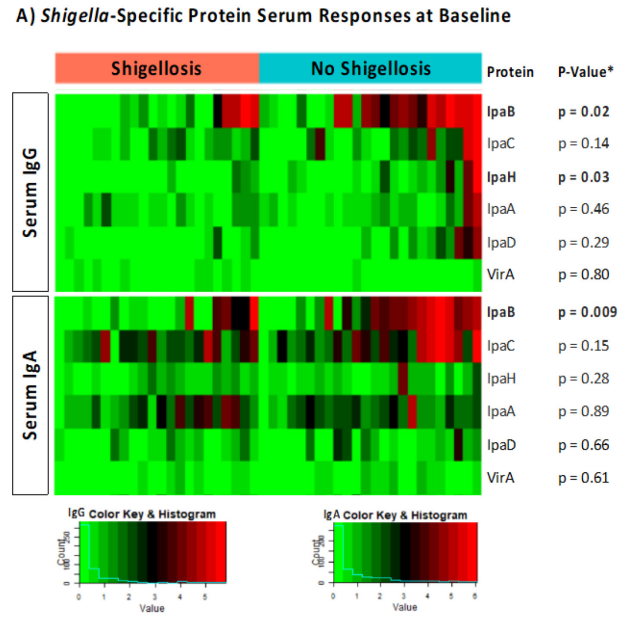
1) Peak titer (T-test of log-transformed titers)
2) Peak fold-rise (Mann-Whitney U test)
3) Percent responders (Mann-Whitney U test)
4) Immune response kinetics (2-way ANOVA of log-transformed titers)

Assay prioritization was assigned as follows:

Primary	= ≥3 measures significantly associated with shigellosis outcome
Secondary	= 1-2 measures significantly associated with shigellosis outcome
Tertiary	= 0 measures significantly associated with shigellosis outcome

Additionally, any baseline immune response significantly associated with resistance to disease after oral challenge was categorized as a primary priority.

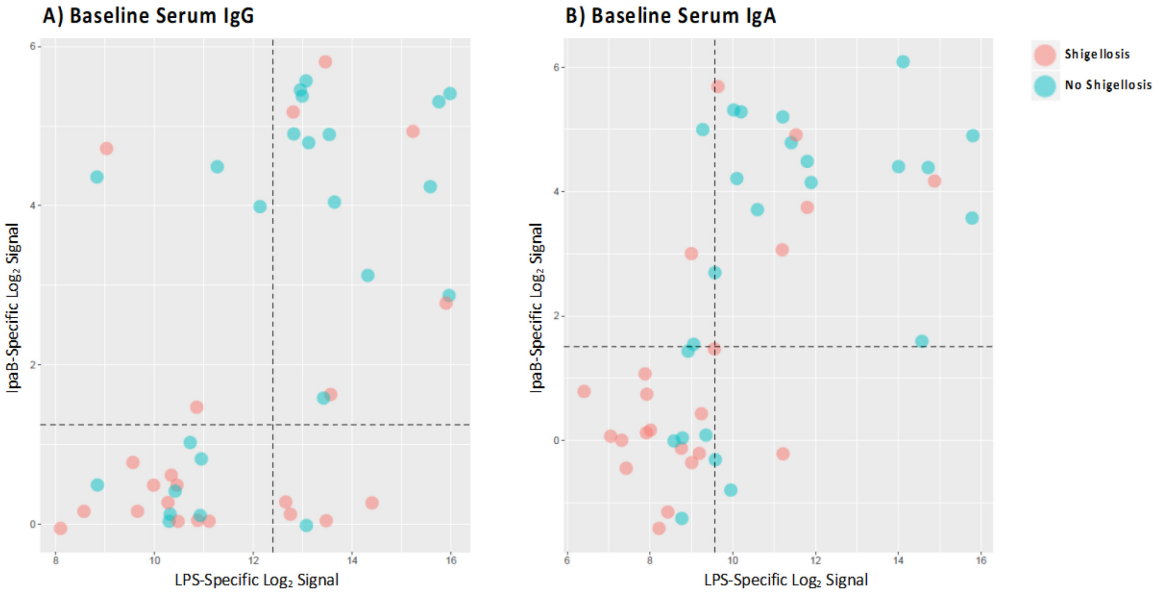
Figure 6.1. IpaB-Specific Baseline Immune Responses in Subjects Orally Challenged with *S. sonnei* 53G



A) Shigella-Specific Protein Serum Responses at Baseline
Shigellosis
No Shigellosis
Protein
P-Value*
Serum IgG
IpaB p = 0.02
IpaC p = 0.14
IpaH p = 0.03
IpaA p = 0.46
IpaD p = 0.29
VirA p = 0.80
Serum IgA
IpaB p = 0.009
IpaC p = 0.15
IpaH p = 0.28
IpaA p = 0.89
IpaD p = 0.66
VirA p = 0.61
IgG Color Key & Histogram
IgA Color Key & Histogram
Count
Value

IpaB-specific immune responses on study day -1 prior to oral challenge with *S. sonnei* 53G: A) Heatmap of microarray immune response signals generated as base 2 logarithm of the raw microarray signal for all samples. * = Significance determined by Wilcoxon rank sum test. Significant p-values (≤0.05) written in bold font. B) Individual IpaB-specific serum IgG ELISA endpoint titers with group geometric mean and 95% confidence interval. Significance determined by T-test of log-transformed titers.

Figure 6.2. Shigellosis Attack Rates in Subjects Orally Challenged with *S. sonnei* 53G based upon *S. sonnei* LPS and IpaB-Specific Serum Antibody Responses Prior to Challenge



	Number of Subjects with Shigellosis/ Total Number in Quadrant (Percent)	
IpaB +	2/5 (40%)	5/18 (28%)
IpaB -	11/18 (61%)	4/5 (80%)
	LPS -	**LPS +**

	Number of Subjects with Shigellosis/ Total Number in Quadrant (Percent)	
IpaB +	1/3 (33%)	5/20 (25%)
IpaB -	15/20 (75%)	1/3 (33%)
	LPS -	**LPS +**

Shigellosis attack rates by quadrant: Serum IgG (A) and IgA (B) microarray immune response signals generated as base 2 logarithm of the raw microarray signal. Dashed lines represent the median value of LPS- and IpaB-specific signals. For the purpose of calculating attack rates in each quadrant without bias in the selection of the thresholds, signals above the median are categorized as positive (+) and below the median are categorized as negative (-). Boxes present attack rates among the subset of subjects in each corresponding quadrant.

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APPENDIX A: ESTABLISHMENT OF A CONTROLLED HUMAN INFECTION MODEL WITH A LYOPHILIZED STRAIN OF *SHIGELLA SONNEI* 53G

The data presented in Appendix A is a companion manuscript to Chapter 3 and has been accepted for publication in the American Society for Microbiology journal mSphere:

Frenck RW, Dickey M, Suvarnapunya AE, Chandrasekaran L, Kaminski RW, Clarkson KA, McNeal M, Lynen A, Parker S, Hoeper A, Mani S, Fix A, Maier N, Venkatesan MM, Porter CK. Establishment of a controlled human infection model with a lyophilized strain of *Shigella sonnei* 53G. *mSphere* 2020; Manuscript Accepted for Publication.

APPENDIX B: HUMAN CHALLENGE STUDY WITH A *SHIGELLA* BIOCONJUGATE VACCINE: ANALYSES OF CLINICAL EFFICACY AND CORRELATE OF PROTECTION

The data presented in Appendix B is a companion manuscript to Chapter 4 and has been submitted for publication to the journal Lancet Infectious Diseases:

Talaat K, Alaimo C, Martin P, Bourgeois AL, Dreyer A, Kaminski RW, Porter CK, Chakraborty S, Clarkson KA, Brubaker J, Elwood D, Frölich R, DeNearing B, Weerts HP, Feijoo B, Halpern J, Sack D, Riddle MS, Gambillara Fonck V. Human challenge study with a *Shigella* bioconjugate vaccine: Analyses of clinical efficacy and correlate of protection. *Lancet Infect Dis* 2020; Manuscript Submitted for Publication.

APPENDIX C: CONSENSUS REPORT ON *SHIGELLA* CONTROLLED HUMAN INFECTION MODEL: IMMUNOLOGICAL ASSAYS

The manuscript attached in Appendix C was published in the journal of Clinical Infectious Diseases in 2019 and is complementary to Chapter 6

This manuscript is a consensus report outlining recommendations for *Shigella* CHIM sample collection schedules and immunological assay prioritizations. This consensus report was developed during the data analysis phases of Chapters 3 and 4 and results generated from these two Chapters helped guide the development of the published recommendations.

Kaminski RW, Pasetti MF, Aguilar AO, Clarkson KA, Rijpkema S, Bourgeois AL, Cohen D, Feavers I, MacLennan CA. Consensus Report on *Shigella* Controlled Human Infection Model: Immunological Assays. Clin Infect Dis. 2019. 69: S596-S601.
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231. Lorin C, Vanloubbeeck Y, Baudart S, et al. Heterologous prime-boost regimens with a recombinant chimpanzee adenoviral vector and adjuvanted F4 protein elicit polyfunctional HIV-1-specific T-Cell responses in macaques. *PLoS One* 2015; **10**(4): e0122835.
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CURRICULUM VITAE

KRISTEN A. CLARKSON

PROFESSIONAL EXPERIENCE

Research Microbiologist **Oct 2016 – Present**
US Department of Defense, Department of the Army
Subunit Enteric Vaccines and Immunology (SEVI) Laboratory
Walter Reed Army Institute of Research (WRAIR), Silver Spring MD

Senior Research Associate **Oct 2015 – Sep 2016**
The McConnell Group
Subunit Enteric Vaccines and Immunology (SEVI) Laboratory
Walter Reed Army Institute of Research (WRAIR), Silver Spring MD

Immunology Lab Manager **Jul 2014 – Sep 2015**
Team Placement Services
Subunit Enteric Vaccines and Immunology (SEVI) Laboratory
Walter Reed Army Institute of Research (WRAIR), Silver Spring MD

Research Associate II **Jul 2012 – Jun 2014**
Team Placement Services
Subunit Enteric Vaccines and Immunology (SEVI) Laboratory
Walter Reed Army Institute of Research (WRAIR), Silver Spring MD

Research Associate I **Jul 2009 – Jun 2012**
Team Placement Services
Invaplex Research Laboratory (IRL)
Walter Reed Army Institute of Research (WRAIR), Silver Spring MD

Student Intern **Jan 2008 – Jun 2009**
Invaplex Research Laboratory (IRL)
Walter Reed Army Institute of Research (WRAIR), Silver Spring MD

RESEARCH EXPERIENCE

Designed assays to investigate immune correlates of protection in *Shigella* infection:
Correlated anti-serotype specific LPS local mucosal IgA production with protection in the guinea pig keratoconjunctivitis *Shigella* challenge model. Induced passive protection in the same model with the use of high titered immune sera. Produced and characterized monoclonal antibodies for assessment in the passive protection model.

Refined a guinea pig intrarectal challenge model:
Designed studies to refine a challenge dose that would induce rectocolitis in guinea pigs weighing ≥450 grams for use in vaccine efficacy studies. Successfully finalized challenge doses for *Shigella flexneri* 2a, *Shigella flexneri* 3a, *Shigella sonnei* and *Campylobacter jejuni*.

Performed *in vitro* characterization of a next-generation subunit *Shigella* vaccine:
Measured the concentration of cytokines released from J774 murine macrophages after incubations with wild type and deacylated *Shigella flexneri* 2a LPS for use in the development of a detoxified next-generation Invaplex subunit vaccine for parenteral administration.

Assisted in the progression of first and second generation *Shigella* subunit vaccines to clinical trials:
Served as project manager for the Phase 1 human clinical trial assessing the safety and immunogenicity of Artificial Invaplex, a second generation *Shigella flexneri* 2a subunit vaccine. Managed all laboratory assay and vaccine formulation aspects of the trial. Assisted the principal investigator in the generation and completion of the FDA required Electronic Common Technical Document (eCTD) and Investigational New Drug (IND) forms. Assisted in the immunological assessments for the Phase 2b human clinical trial assessing the immunogenicity and efficacy of Invaplex 50, a first generation *Shigella flexneri* 2a subunit vaccine.

Assessed immune responses induced by a novel bioconjugate vaccine in a Phase 1 clinical trial:
Served as project manager for the Phase 1 human clinical trial assessing the safety and tolerability of a candidate *Shigella flexneri* 2a bioconjugate vaccine formulated with and without an adjuvant. Assessed immunological response data in volunteers and compiled and analyzed the data for presentation at the Vaccines for Enteric Diseases conference, 2015.

Developed multiplex assay to measure the immune response to several antigens simultaneously:
Developed Luminex-based assay to simultaneously measure the antibody responses to six different *Shigella* antigens. Refined the assay for use with serum, whole blood eluted from filter paper and mucosal washes from several different species.

Pre-clinical development and characterization of a whole cell inactivated *Shigella* vaccine:
Assisted in the optimization of inactivating *Shigella flexneri* 2a, *Shigella flexneri* 3a and *Shigella sonnei* for the use as whole cell inactivated vaccines. Designed small animal pre-clinical studies to determine the optimal dose, route of immunization and benefits of an adjuvant.

Development of an *in vitro* assay to study the interaction between M cells and mucosal pathogens:
Optimized the development of a differentiated cell monolayer in a transwell system and monitored conversion of Caco-2 epithelial cells to M cells. Assessed the ability of *Shigella* to use the M cells to transcytose from the apical side to the basolateral side of the transwell. Evaluated the antibody-mediated inhibition of *Shigella* transcytosis across the monolayer.

EDUCATION

Johns Hopkins Bloomberg School of Public Health, Baltimore MD **Graduated Aug 2020**

Doctor of Philosophy

- Thesis Title: "*Shigella*-Specific Protective Immune Profiles Induced after Parenteral Immunization or Oral Challenge with Two Different *Shigella* Serotypes"
- GPA: 3.7

Johns Hopkins University, Baltimore MD **Graduated May 2015**

Master of Science in Biotechnology

- GPA: 3.9

George Mason University, Fairfax VA **Graduated May 2009**

Bachelor of Science in Biology with Honors (Minor in Chemistry)

- GPA: 3.7
- Dean's List Recipient (2007, 2008, 2009)

PUBLICATIONS

1. **Clarkson, K.A.**, Talaat, K.R., Alaimo, C., Martin, P., Bourgeois, A.L., Dreyer, A., Porter, C.K., Chakraborty, S., Brubaker, J., Elwood, D., Frölich, R., DeNearing, B., Weerts, H., Feijoo, B., Halpern, J., Sack, D., Riddle, M.S., Gambillara Fonck, V., Kaminski, R.W. (2020). Immune Response Characterization in a Human Challenge Study with a *Shigella flexneri* 2a Bioconjugate Vaccine. Lancet Infectious Diseases. Manuscript Submitted for Publication.

2. Talaat, K.R., Alaimo, C., Martin, P., Bourgeois, A.L., Dreyer, A., Kaminski, R.W., Porter, C.K., Chakraborty, S., **Clarkson, K.A.**, Brubaker, J., Elwood, D., Frölich, R., DeNearing, B., Weerts, H., Feijoo, B., Halpern, J., Sack, D., Riddle, M.S., Gambillara Fonck, V. (2020). Human challenge study with a *Shigella* bioconjugate vaccine: Analyses of clinical efficacy and correlate of protection. Lancet Infectious Diseases. Manuscript Submitted for Publication.

3. **Clarkson, K.A.**, Frenck, R.W., Dickey, M., Suvarnapunya, A.E., Chandrasekaran, L., Weerts, H.P., Heaney, C.D., McNeal, M., Detizio, K., Parker, S., Hoeper, A., Bourgeois, A.L., Porter, C.K., Venkatesan, M., Kaminski, R.W. (2020). Immune Response Characterization after Controlled Infection with Lyophilized *Shigella sonnei* 53G. mSphere. Manuscript Accepetd for Publication.

4. Frenck, R.W., Dickey, M., Suvarnapunya, A.E., Chandrasekaran, L., Kaminski, R.W., **Clarkson, K.A.**, McNeal, M., Lynen, A., Parker, S., Hoeper, A., Mani, S., Fix, A., Maier, N., Venkatesan, M., Porter, C.K. (2020). Establishment of a controlled human infection model with a lyophilized strain of *Shigella sonnei* 53G. mSphere. Manuscript Accepetd for Publication.

5. Kaminski, R.W., Pasetti, M.F., Aguilar, A.O., **Clarkson, K.A.**, Rijpkema, S., Bourgeois, A.L., Cohen, D., Feavers, I., MacLennan, C.A. (2019). Consensus Report on *Shigella* Controlled Human Infection Model: Immunological Assays. Clinical Infectious Diseases, 69(Supplement_8): S596-S601.

6. Turbyfill, K.R., **Clarkson, K.A.**, Vortherms, A.R., Oaks, E.V., Kaminski, R.W. (2018). Assembly, Biochemical Characterization, Immunogenicity, Adjuvanticity, and Efficacy of *Shigella* Artificial Invaplex. mSphere, 3(2): e00583-17.

7. Wenzel, H., Kaminski, R.W., **Clarkson, K.A.**, Maciel, M. Jr, Smith, M.A., Zhang, W., Oaks, E.V. (2017). Improving chances for successful clinical outcomes with better preclinical models. Vaccine, 14;35(49 Pt A):6798-6802.

8. Riddle, M.S., Kaminski, R.W., Di Paolo, C., Porter, C.K., Gutierrez, R.L., **Clarkson, K.A.**, Weerts, H.E., Duplessis, C., Castellano, A., Alaimo, C., Paolino, K., Gormley, R., Gambillara Fonck, V. (2016). Safety and Immunogenicity of a Candidate Bioconjugate Vaccine against *Shigella flexneri* 2a Administered to Healthy Adults: A Single-Blind, Randomized Phase I Study. Clinical and Vaccine Immunology: CVI, 23(12), 908–917.

9. Kaminski, R. W., Wu, M., Turbyfill, K. R., **Clarkson, K.**, Tai, B., Bourgeois, A. L., Van De Verg, L.L., Walker, R.I., Oaks, E. V. (2014). Development and Preclinical Evaluation of a Trivalent, Formalin-Inactivated *Shigella* Whole-Cell Vaccine. Clinical and Vaccine Immunology: CVI, 21(3), 366–382.

10. Kaminski, R.W., **Clarkson, K.**, Kordis, A.A, Oaks, E.V. (2013). Multiplexed immunoassay to assess *Shigella*-specific antibody responses. Journal of Immunological Methods. 393(1–2), 18-29.

ORAL PRESENTATIONS

1. **K.A. Clarkson**, R. Gutierrez, K.R. Turbyfill, K. Detizio, A.R. Vortherms, A. Lynen, B. Barnard, H. Weerts, C.K. Porter, N. Maier, R. Erdem, A.L. Bourgeois, R.W. Kaminski. GMP Manufacture, Characterization and Clinical Evaluation of *Shigella flexneri* 2a Detoxified Artificial Invaplex. Vaccines for Enteric Diseases (VED) Conference, Lausanne, Switzerland. October 2019.

2. **K.A. Clarkson**, R.W. Frenck Jr, M. Dickey, A.E. Suvarnapunya, L. Chandrasekaran, K.T. Lerner, B.A. Barnard, H.P. Weerts, M. McNeal, K. Detizio, S. Parker, A. Hoeper, S. Mani, C.K. Porter, N. Maier, A. Fix, A. L. Bourgeois, M. Venkatesan, R.W. Kaminski. Immune Response Characterization after Controlled Infection with a Lyophilized *Shigella sonnei* 53G, (cGMP Lot 1794). Vaccines Against Shigella and ETEC (VASE) Conference, Mexico City, Mexico, June 2018.

3. C.K. Porter, A.M. Dreyer, C. Alaimo, P. Martin, R.W. Kaminski, **K.A. Clarkson**, A.L. Bourgeois, D. Sack, S. Chakraborty, J. Brubaker, K. Talaat, V. Gambillara Fonck. Immune response profiles following vaccination with a *Shigella* bioconjugate vaccine that correlate with a reduction in shigellosis severity. Vaccines Against Shigella and ETEC (VASE) Conference, Mexico City, Mexico, June 2018.

4. **K.A. Clarkson**, C. Duplessis, K.R. Turbyfill, C. Porter, R. Gutierrez, M. S. Riddle, T. Lee, H.E. Weerts, S.C. Sumlin, A. Lynen, E.V. Oaks, K. Paolino, G. Fornillos, R.W. Kaminski. A Phase 1 Open-label, Dose Escalating Study of *Shigella flexneri* 2a Artificial Invaplex administered intranasally to healthy, adult volunteers. Vaccines for Enteric Diseases (VED) Conference, Albufeira, Portugal. October 2017.

5. K.R. Talaat, C. Alaimo, A.L. Bourgeois. R.W. Kaminski, A. Dreyer, C.K. Porter, S. Chakraborty, **K.A. Clarkson**, J. Brubaker, D. Elwood, R. Frölich, B. DeNearing, H.P. Weerts, B. Feijoo, J. Halpern, D. Sack, M.S. Riddle, P. Martin, and V. Gambillara Fonck. Flexyn2a, a candidate bioconjugate vaccine against *Shigella flexneri* 2a induces protective immune response in a controlled human infection model. Vaccines for Enteric Diseases (VED) Conference, Albufeira, Portugal. October 2017.

6. C. Duplessis, K.R. Turbyfill, C. Porter, R. Gutierrez, M. S. Riddle, T. Lee, **K.A. Clarkson**, H.E. Petersen, C.R. Stelez, S.C. Sumlin, A. Lynen, E.V. Oaks, K. Paolino, W. Fornillos, and R.W. Kaminski. A Phase 1 Open-label, Dose Escalating Study of Artificial *Shigella flexneri* 2a Invaplex administered intranasally to healthy, adult volunteers. Vaccines against Shigella and ETEC (VASE) Conference, Washington DC, USA. June 2016.

7. M. Riddle, R.W. Kaminski, C. Di Paolo, C. Porter, R. Gutierrez, **K.A. Clarkson**, H.E. Petersen, C. Duplessis, A. Castellano, K. Paolino, R. Gormley, V. Gambillara. Safety and immunogenicity of a candidate bioconjugate vaccine against *Shigella flexneri* 2a administered to healthy adults: a single blind, randomized phase I study. Vaccines against Shigella and ETEC (VASE) Conference, Washington DC, USA. June 2016.

8. M. Riddle, R. Kaminski, C. Di Paolo, C. Porter, R. Gutierrez, K. Paolino, **K. Clarkson,** C. Duplessis, C. Soltis, C. Strelez, K. Jaep, A. Castellano, M. Wacker and V. Gambillara. Clinical evaluation of the *S. flexneri* bioconjugate vaccine. Vaccines for Enteric Diseases (VED) Conference, Edinburgh, Scotland. July 2015.

INVITED TALKS

1. **Kristen A. Clarkson**. Host Immune Responses after Oral *Shigella* Challenge: US-Based *Shigella sonnei* Human Infection Studies. Wellcome Trust. Kilifi, Kenya, November 2019.

2. **Kristen A. Clarkson**. Achieving Mucosal Protection: Human *Shigella* Challenge Studies After Vaccination with a *Shigella* Conjugate Vaccine. Vaccines Against Shigella and ETEC (VASE) Conference, Mexico City, Mexico, June 2018.

3. **Kristen A. Clarkson**. *Shigella* International ELISA: *Shigella* O-Antigen ELISA Experiences and Protocols. Bill and Melinda Gates Foundation: *Shigella* International ELISA Workshop, London, UK, December 2017.

4. **Kristen A. Clarkson**. *Shigella* CHIM Immunoassays: Assessment of Immune Responses After Controlled Infection with *Shigella* spp. Bill and Melinda Gates Foundation: *Shigella* CHIM Endpoints and Immunoassays Workshop, Washington DC, USA, November 2017.

5. **Kristen A. Clarkson**. Immunoassays: How we Assess *Shigella* Vaccine Immune Responses. Bill and Melinda Gates Foundation: *Shigella* Vaccinology Workshop, Seattle WA, USA, September 2017.

6. **Kristen A. Clarkson**. Guinea Pig *Shigella* Challenge Models and their use in Vaccine Efficacy Studies Vaccines Against Shigella and ETEC (VASE) Conference, Washington DC, USA. June 2016.

POSTER PRESENTATIONS

1. B.A. Barnard, **K.A. Clarkson**, R.W. Frenck Jr, M. Dickey, A.E. Suvarnapunya, L. Chandrasekaran, K.T. Lerner, H.P. Weerts, M. McNeal, K. Detizio, S. Parker, A. Hoeper, S. Mani, C.K. Porter, N. Maier, A. Fix, A. L. Bourgeois, M. Venkatesan, R.W. Kaminski. Antigen-Specific Gut-Homing B Cell Responses in Humans Following Controlled Infection with Lyophilized *Shigella sonnei* 53G, (cGMP Lot 1794). American Society of Tropical Medicine and Hygiene (ASTMH) Conference, National Harbor, Maryland USA, November 2019.

2. S. Rose, C. Carter, K. Nelson, **K.A. Clarkson**, J. Justen, and H. Wenzel. Preclinical Safety Assessment of a Detoxified Lipopolysaccharide-Based *Shigella flexneri* 2a Vaccine Candidate in New Zealand White Rabbits. European Society of Clinical Microbiology and Infectious Diseases (ECCMID), Amsterdam, Netherlands, April 2019.

3. C.R. Dunbar, M.S. Riddle, **K.A. Clarkson**, R.L. Gutierrez, A.N. Alcala, A. Byrd, and C.K. Porter. Deployment-Associated Infectious Gastroenteritis and Associations with Irritable Bowel Syndrome, Post-Traumatic Stress Disorder, and Combat Stress: A Retrospective Cohort Study Among Deployed United States Military Personnel. Infectious Disease Society of America (IDSA) Annual Meeting. San Francisco, California USA. October 2018.

4. **K.A. Clarkson**, A.M. Dreyer, C. Alaimo, K.R. Talaat, B.A. Barnard, K.T. Lerner, V. Gambillara Fonck, P. Martin, R.W. Kaminski. Parenteral Immunization with the *Shigella flexneri* 2a Bioconjugate Vaccine Induces LPS-Specific Memory B Cell Responses. Vaccines Against Shigella and ETEC (VASE) Conference, Mexico City, Mexico, June 2018.

5. K.R. Talaat, C. Alaimo, A.L. Bourgeois. R.W. Kaminski, A. Dreyer, C.K. Porter, S. Chakraborty, **K.A. Clarkson**, J. Brubaker, D. Elwood, R. Frölich, B. DeNearing, H.P. Weerts, B. Feijoo, D. Sack, M.S. Riddle, P. Martin, and V. Gambillara Fonck. Flexyn2a, A Candidate Bioconjugate Vaccine Against *Shigella flexneri* 2a Induces Protective Immune Response in a Controlled Human Infection Model. American Society of Tropical Medicine and Hygiene (ASTMH) Conference, Baltimore, Maryland USA. November 2017.

6. **K.A. Clarkson**, A.M. Dreyer, K.R. Talaat, C. Alaimo, A.L. Bourgeois, C.K. Porter, S. Chakraborty, J. Brubaker, D. Elwood, R. Frölich, B. DeNearing, H.P. Weerts, B. Feijoo, J. Halpern, D. Sack, M.S. Riddle, V. Gambillara Fonck, P. Martin, R.W. Kaminski. Parenteral immunization with the *Shigella flexneri* 2a bioconjugate vaccine induces gut-homing LPS-specific antibody secreting cells. Vaccines for Enteric Diseases (VED) Conference, Albufeira, Portugal. October 2017.

7. A.M. Dreyer, **K.A. Clarkson**, H.P. Weerts, C. Alaimo, K.R. Talaat, A.L. Bourgeois, C.K. Porter, S. Chakraborty, J. Brubaker, D. Elwood, R. Frölich, B. DeNearing, B. Feijoo, J. Halpern, D. Sack, M.S. Riddle, V. Gambillara Fonck, R.W. Kaminski, P. Martin. Systemic LPS-specific IgG responses to *Shigella* bioconjugate vaccine, correlate with protection against infection with *Shigella flexneri* 2a in a human challenge model. Vaccines for Enteric Diseases (VED) Conference, Albufeira, Portugal. October 2017.

8. M.A. Smith, K. Hinton, B. McKinney, **K.A. Clarkson**, R.W. Kaminski. Protection against *Shigella* ocular challenge in previously infected guinea pigs: Potential model for deciphering immune correlates of protection. American College of Veterinary Pathologists Conference, New Orleans, Louisiana USA. December 2016.

9. **K.A. Clarkson**, P. Erlich, M.A. Smith, G. Nigro, D. Poncet, K.R. Turbyfill, F. Raynal, P. Chaux, P. Sansonetti, A. Phalipon, E.V. Oaks, G. Renauld-Mongénie, and R.W. Kaminski. Refinement of a Guinea Pig Intrarectal *Shigella* Challenge Model and use in Vaccine Efficacy Studies. Vaccines against Shigella and ETEC (VASE) Conference, Washington DC, USA. June 2016.

10. R.W. Kaminski, **K.A. Clarkson**, K.R. Turbyfill, M.A. Smith, C.R. Strelez, A.R. Vortherms, D. Jirage, H. Wenzel, L. Van De Verg, R. Walker, J.D. Clements, and E.V. Oaks. Evaluation of *Shigella flexneri* 2a Artificial Invaplex Formulated for Parenteral Immunization with Deacylated LPS. Vaccines against Shigella and ETEC (VASE) Conference, Washington DC, USA. June 2016.

11. R.M. Laird, S. Shahabudin, **K.A. Clarkson,** R.W. Kaminski, S.J. Savarino, M.R. Riddle, R.L. Gutierrez and M. Maciel Jr. Memory B cells with gut-homing potential are generated by a prototype anti-enterotoxigenic *E. coli* vaccine given by intradermal immunization with LT(R192G) as adjuvant. Immunology 2015 Conference, New Orleans, Louisiana USA. May 2015

12. M.A. Smith, R.W. Kaminski, **K.A. Clarkson,** T. Pierson, N. Bryant, M. Marll, M. Pratt, K.R. Turbyfill and E.V. Oaks. Non-GLP mouse toxicology studies of *Shigella* Invaplex$_{AR}$ vaccine delivered via intradermal and intranasal routes. 13th Annual Conference on Vaccine Research, Bethesda, Maryland USA. May 2014.

13. R.W. Kaminski, **K.A. Clarkson,** M.L. Buck, A.A. Kordis, and E.V. Oaks. Multiplex Assay for Simultaneous Measurement of Antibodies to Multiple *Shigella* Antigens. American Society of Microbiology (ASM) General Meeting, San Diego, California USA. May 2010.

14. R.W. Kaminski, A.A. Kordis, M.L Buck, **K.A. Clarkson,** and E.V. Oaks. Antibody-Mediated Inhibition of *Shigella flexneri* 2a across an *in vitro* M Cell Model. American Society of Microbiology (ASM) General Meeting, Philadelphia, Pennsylvania USA. May 2009.

15. T.L. Boren, S.M. Wallace, J.E. Lee, S.L. Fonseka, R.W. Kaminski, **K.A. Clarkson,** M.M. Venkatesan, T.L. Hale, G.L. Gustafson, D.C. DeBorde, and R.T. Ranallo. Development of a Nonlethal Intravenous Murine Challenge Model: Potential for Use in *Shigella* Pathogenesis & Vaccine Efficacy Studies. American Society of Microbiology (ASM) General Meeting, Philadelphia, Pennsylvania USA. May 2009.

FUNDED RESEARCH PROPOSALS

- US Army Medical Infectious Disease Research Program (MIDRP). MIDRP D0461_16_WR. "Determination of immune correlates of protection for infection with *Shigella* species." Role: Associate Investigator, $142,000.

- US Army Medical Infectious Disease Research Program (MIDRP). MIDRP D0520_17_WR. "Evaluation and Characterization of *Shigella* Vaccines and Products." Role: Associate Investigator, $410,000.

PATENTS

- Artificial Invaplex Vaccine Formulated with Deacylated Lipopolysaccharide. Inventors: Robert W. Kaminski, Kevin R. Turbyfill, Kristen A. Clarkson and Edwin V. Oaks. Submitted for International PCT.